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# Temporal Variation in Larval Fish Condition Indices.

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TEMPORAL VARIATION IN LARVAL FISH  
CONDITION INDICES

A Dissertation

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Doctor of Philosophy

in

The Department of Oceanography and Coastal Sciences

by

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B.S., Oregon State University, 1986  
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May, 1998

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## ABSTRACT

A sensitive methodology for the determination of RNA and DNA concentrations was developed which allowed for the quantification of nanogram amounts of both DNA and RNA. The method uses a combination of thiazole orange, a non-specific nucleic acid stain, and the RNA degrading enzyme RNase. Thiazole orange had high fluorescence yield. The fluorescence was additive for combinations of RNA and DNA. The addition of proteinase-K increased the fluorescence of the tissue sample by 100%. Recovery of nucleic acid standards added to tissue extracts yielded ~100% recovery. The method is compatible with assays for protein concentration and electron transport system (ETS) enzyme activity.

Menhaden (*Brevoortia patromus*) larvae and meteorological data were collected for 2 week intervals during the winters months of 1993-1994 and 1994-1995 in an examination of the role of atmospheric frontal passage on larval condition. Three biochemical assays were used to quantify larval condition, RNA:DNA, protein concentration and ETS activity. Each day of collection was categorized in terms of the prevailing atmospheric conditions.

The larvae were in good condition and there was no consistent trend in the condition of the larvae through the two spawning season. There was no indication that poor condition larvae were being lost from the population. Larval condition was not strongly affected by water temperature or any meteorological variable. All condition indices were highest during periods of southerly winds associated with the progression of atmospheric cold fronts. The variation in condition indices among the months of

collection and between the years of collection can be explained by the frequency of the atmospheric cold fronts. The increase in larval condition in association with the cold fronts is likely a result of the increased encounter rate with prey items due to an increase in wind-generated turbulence.

A new morphometric analysis technique based upon the thin-plate spline was examined as a low-cost alternative to the biochemical measures of condition. This method of shape analysis was not successful in identifying the known condition of the larvae. The short-term influence of the atmospheric cold fronts was of insufficient intensity to generate morphological change in the larvae.

## **CHAPTER 1: INTRODUCTION**

Populations of marine fish undergo dramatic fluctuations in year-class strength. These seemingly random fluctuations are problematic for fisheries managers that need to regulate the fishery on the basis of predicted population size. It is generally believed that the greatest influence on the size of each year class is the number of individuals that survive the larval stage. Three major processes that affect survival through the larval stage are predation, feeding success and transport to juvenile nursery areas. It is likely that these three mechanisms act in concert in determining the number of individuals that survive through the larval stage.

### **FEEDING SUCCESS**

By far, the greatest volume of work related to larval fish ecology examines the effect of feeding success on survival. Early work was based on the observation that the concentrations of prey items required for larval growth and survival in the laboratory were much greater than prey concentrations observed in the field. It was concluded that starvation, especially associated with first feeding larvae (the “critical period”), greatly impacted larval survival. However, field studies only rarely showed evidence of widespread starvation (see Theilacker, 1986, Grover and Olla, 1986). Many early laboratory studies employed easily cultured prey items such as rotifers or *Artemia* (e.g. Goshorn and Epifanio, 1991) which do not support the rapid growth observed in larvae fed natural zooplankton (Taniguchi, 1981). Additionally, laboratory estimates of growth in relation to prey density failed to incorporate other factors that are now thought to influence larval feeding success in the wild, such as the patchy distributions of prey and

larvae (Mackenzie et al., 1990) and turbulence that would influence encounter rates between larvae and their prey (Rothschild and Osborn, 1988).

Although direct starvation alone does not appear to be a significant contributor to the determination of year class strength, the reduced growth rates and reduced behavioral performance of larvae that are feeding at sub-optimal levels will still impact larval survival. The reduced growth rate will prolong the larval duration subjecting the larvae to a larger suite of predators for a greater period of time (Houde, 1989). Yin and Blaxter (1987) demonstrated that the escape response of various species of fish larvae was significantly reduced after poor feeding conditions, and suggested that a poorly fed larvae will be more susceptible to predation. Additionally, larvae in poor nutritional states fail to respond to environmental stimuli (e.g. light, gravity) in a manner consistent with the response of well-fed larvae (Davis and Olla, 1992). This differential response to environmental stimuli can result in the vertical separation of the larvae in the water column and therefore differential transport via surface currents (Sclafani et al., 1993; Power, 1984).

### **LARVAL TRANSPORT**

A second factor important for larval survival in many species of marine fish appears to be transport to estuarine or near-shore nursery grounds (Boehlert and Mundy, 1988). Typically the adults move into the estuary or the near-shore waters to spawn. The resulting young are then retained in the estuary, invade the estuary or move near shore during the larval stage. However, the net seaward flow of estuaries creates an environment not conducive to recruiting or retaining weakly swimming larval stages.



Two general mechanisms have been proposed to account for the large-scale movements of larvae: behaviorally mediated transport and passive transport via wind-generated currents. It is likely that both behavioral and passive mechanisms, acting in concert, regulate the onshore and estuarine transport of both invertebrate and vertebrate larvae (Norcross and Shaw, 1984; Boehlert and Mundy, 1988).

Active vertical migrations are believed to be commonly used by larvae to affect their horizontal transport to favorable locations. The majority of field and laboratory data confirm that the vertical distribution of fish and invertebrate larvae is a result of active vertical migrations on the part of the larvae (Weinstein et al., 1980; McCleave and Kleckner, 1982; Fortier and Leggett, 1983; Boehlert and Mundy, 1987; Castonguay and McCleave, 1987, Little and Epifanio 1990). Because currents vary with depth (Sulkin et al., 1980; Pape and Garvine, 1982 ), vertical distribution then becomes the main determining factor in the transport and retention of the larvae. Power (1997) demonstrated that a nocturnally active zooplankter can achieve seasonally consistent upstream transport in several geographically diverse estuarine systems.

In addition to vertical migrations, episodic or seasonal winds have also been hypothesized as important for the transport of larvae into estuaries. Along the Atlantic and Gulf coasts, the progression of atmospheric frontal passages occurs with regularity and can be utilized as a stable feature for invasion of an estuary. The winds associated with these atmospheric fronts generate surface currents that can transport oceanic water and associated plankton into the estuary. This influx of oceanic water is often referred to as a storm surge.

For example, Goodrich et al. (1989) found that the large pulses of blue crab (*Callinectes sapidus*) megalopae entering Chesapeake Bay were temporally associated with the storm surges resulting from the progression of atmospheric fronts. Rogers et al. (1993) hypothesize that brown shrimp (*Penaeus aztecus*) larvae utilize temperature and salinity cues to modify their vertical distribution so as to facilitate transport via the wind generated shoreward currents associated with atmospheric fronts in the Gulf of Mexico. Menhaden (*Brevoortia* sp.) along both the Gulf and Atlantic coasts are thought to depend on frontal passage for transport to estuarine nursery areas ( Shaw et al. 1985; Checkley et al., 1988).

## PREDATION

Because of their relatively small size and limited mobility, marine fish larvae are susceptible to a large number of predators, and their vulnerability to predators depends directly on their recent success in both feeding and transport. Potential predators of larval fish and eggs include copepods (Lillelund and Lasker, 1971), various species of gelatinous zooplankton (Purcell, 1985), chaetognaths (Brewer et al., 1984), shrimp (Yamashita et al., 1996) and large numbers of vertebrate juveniles and adults (Webb, 1981; Gamble and Fuiman, 1987; Fuiman, 1989; Fuiman and Batty, 1994). Cushing (1975) suggested that an inverse relationship exists between mortality and larval size, and that this relationship results from the reduced suite of potential predators as larval size increases. In other words, well-fed, fast-growing larvae are able to escape predators that can only consume the smaller larvae.

The ability of an organism to consume larval fish and eggs in a laboratory setting does not necessarily imply the predators potential impact on the natural larval population. The predator and prey must co-occur both temporally and spatially. Predator-avoidance mechanisms can be behavioral or a result of the life history of the organism. Behavior modifications include vertical migrations within the water column and estuarine habitat choice. Vertical migrations out of the surface waters during the night may remove the larvae from the prey field of surface filter feeders (Bailey and Houde, 1989). As they enter the estuary, menhaden larvae migrate into the tidal creeks and bayous. This serves to remove them from the greater concentration of predators in the open bays and their small size limits the effect of avian predation (Deegan, 1985, Baltz et al., 1993). Life- history modifications include the production of eggs and larvae prior to the peak occurrence of the potential predators. de Lafontaine and Leggett (1988) suggested that the year class strength of capelin (*Mallotus villosus*) was regulated by the seasonal appearance and growth of gelatinous predators, emphasizing importance of both the temporal overlap and appropriate predator-prey size relationships.

### CONDITION INDICES

It is apparent from the above considerations that the survival of marine larvae is dependent upon the inter-relationship of feeding, predation and transport. While the starvation of individuals is apparently rare, a well-fed or better-conditioned larva will be better able to avoid predation (Yin and Blaxter, 1987) and respond appropriately to environmental stimuli which will position the larvae for favorable transport (Davis and Olla, 1992; Rogers et al., 1993). Several types of condition indices have been developed

and utilized in an effort to quantify the relative health of larvae. These indices are used to examine the affect of various environmental and anthropogenic stressors on the growth and survival of the larvae. The efficiency of numerous condition indices and their application to marine fisheries was reviewed by Ferron and Leggett (1994). Condition indices can be separated into 2 broad categories: morphological and biochemical.

### **Morphological Indices**

Larvae growth and development is rapid and coupled with dramatic alterations in the organisms shape and proportionality. It has been widely demonstrated that poorly fed larvae do not exhibit the same developmental shape changes in overall body measurements compared to well-fed larvae (Theilacker, 1986; Powell et al., 1990). Morphological indices are based on the premise that some aspects of larval shape are unaffected by reduced food concentrations (e.g. length, eye diameter) while other measurements will reflect the recent feeding and growth (e.g. body depth, weight). A morphometric-condition index is calculated by comparing the feeding-dependent measurement to a feeding-independent measurement. Typically, morphometric indices are relatively low cost, rapid measures of condition, but they are limited in their ability to detect short-term variation because of the low sensitivity to feeding changes.

### **Biochemical Indices**

Several biochemical-condition indices that examine the growth activity at a cellular level have been developed. Among these are measures of RNA and protein concentration and enzyme activity. Since fish larvae are > 50% protein by dry weight

and proteins can be catabolized during times of stress, the total concentration of protein can be an indicator of growth and starvation. RNA, which is the template used for biochemical manufacturing, can be used as an estimate of protein production in the cell, a precursor to growth. Both metabolic and digestive enzymes have also been successfully employed as indicators of condition, however digestive enzymes are influenced greatly by gut contents and have limited utility (Ferron and Leggett, 1993). Although most biochemical indices are highly sensitive and accurate predictors of condition over all time scales, the higher cost and processing time can limit the application in certain circumstances.

The purpose of this study was to examine the condition of larval gulf menhaden (*Brevoortia patronus*) in relation to abiotic episodic events (atmospheric fronts) as the larvae are recruited to the estuary by using proven biochemical condition measures and a new morphometric technique. This species is ideal for this purpose, because of the larvae's relationship to atmospheric frontal passages with respect to both its inshore transport and to frontal effects on the larval food supply.

### **MENHADEN FISHERY**

Menhaden are a ubiquitous, ecologically important species in the Gulf of Mexico supporting a large commercial fishery. Gulf menhaden are distributed throughout the Gulf of Mexico from the Yucatan peninsula to Tampa Bay, Florida with the largest concentrations off the coast of Louisiana. Menhaden support one of the largest commercial fisheries in the United States (Lassuy, 1983) and have accounted for as much as 29% of the total U.S. landings of fish and shellfish by weight (Leard et al., 1995).

The fishery is focused primarily on age 1 fishes (Vaughan et al., 1995). The fishery is dominated by the reduction fishery which produces fish meal, fish oil and condensed fish solubles. A small bait fishery also exists and can account for 2% of the total landings.

### **LIFE HISTORY**

Adult menhaden spawn during the late fall and early winter months with the greatest concentration of spawning occurring off the coast of Louisiana during December (Shaw et al., 1985). Average fecundity is 23,000 eggs per mature female (Vaughan, 1987). The eggs are 1.0-1.3 mm in diameter and buoyant (Houde and Fore, 1973). Time to hatch is temperature dependent; at 19° - 20° C incubation is approximately 40 h (Hettler, 1984). At hatch, the larvae are 2.6 to 3.0 mm standard length (SL) (Hettler, 1984), have non-functional eyes and an undeveloped mouth (Houde and Fore, 1973). Yolk-sac absorption is complete in 80 h at a length of 4.5 mm (Hettler, 1984). Oceanic larvae are particulate feeders with the young larval (<5.0 mm SL) diet comprising dinoflagellates, invertebrate eggs and tintinnids. Larger larvae (5.0-15 mm SL) rely more heavily on copepods (adults, copepodites and nauplii) (Govoni et al., 1983). The gut content of larger larvae were unidentifiable but contained diatom and dinoflagellate fragments (Govoni et al., 1983). Shaw et al. (1988) reported that menhaden larvae spend 3-5 weeks in the near shore water prior to moving into the estuary. Deegan (1985) reported a longer (6-10 week) oceanic period. Peak migration into the estuarine system occurs between December and March (Lassuy, 1983). Once in the estuarine system, the larvae move into the tidal creeks. With the marsh-edge serving as a predator refuge and the abundant food of the estuary, growth is rapid and mortality

is low (Deegan, 1990, Baltz et al., 1993). Metamorphosis to the juvenile stage begins at 20 mm SL and is usually complete by 30 mm (Hettler, 1984). During metamorphosis, growth in length is reduced as body depth increases (Raynie, 1991; Hettler, 1984). It is also at this time that menhaden switch from particulate feeders to omnivorous filter feeders (Govoni et al., 1983; Deegan, 1985). Early juveniles move out of the tidal creeks and into the open bay areas of the estuary in April-June, co-incident with the spring increase in primary production in the open waters (Deegan, 1990). Offshore movement of the young of the year begins in August at 80 to 130 mm SL.

### PHYSICAL OCEANOGRAPHY

The dominant feature of the Louisiana continental shelf is the plume of the Mississippi River. The Mississippi River outflow ranges from  $3000 \text{ m}^3 \text{ sec}^{-1}$  to  $9000 \text{ m}^3 \text{ sec}^{-1}$  with peak flows occurring during the spring. The plume exiting the Southwest Pass drifts in an anticyclonic path, as it drifts shoreward it separates into eastward and westward flowing components (Wiseman et al., 1976). The anthropogenically enhanced nutrient load of the plume waters promotes increased primary production. The enhanced productivity in association with the summertime stratification produces large scale, seasonal hypoxia of bottom waters which dissipates in response to the increased winds of the fall and winter months (Rabalais et al., 1991). West of the delta, the near shore physical oceanography is characterized by an elongated cyclonic gyre which extends from  $92.5^\circ \text{ W}$  to the Texas coast (Cochrane and Kelley, 1986). The gyre is driven by stress associated with alongshore winds and dissipates in July and August as the winds shift (Cochrane and Kelley, 1986). Between  $92.5^\circ \text{ W}$  and the Mississippi River delta, the

shallow coastal waters show only a weak coherence with alongshore winds (Chuang and Wiseman, 1983). In these shallower waters, the coastal currents are driven by cross-shelf winds (Chuang and Wiseman, 1983).

The Louisiana coastline is characterized by numerous, shallow, highly productive estuaries and associated wetlands. The estuaries serve as an important nursery areas for many species of estuarine dependent marine invertebrates and vertebrates including menhaden. It has been estimated that 98% of Louisiana fisheries rely on species that use the estuaries for some portion of their life history (McHugh, 1967). Due to the shallow depth and high surface-to-volume ratio, the ocean-estuary water exchange of these estuaries is dominated by meteorological forcing. The amplitude of the diurnal astronomical tides is 0.3-0.7 m and can be over ridden by the episodic frontal passage (Ward, 1980). Local sea level change due to atmospheric forcing can be up to three times the maximum tidal range (Chuang and Wiseman, 1983).



## **CHAPTER 2: NUCLEIC ACID METHOD DEVELOPMENT**

### **INTRODUCTION**

The concentration of nucleic acids has been utilized extensively to indicate condition in larval and juvenile fish, phytoplankton and marine invertebrates. The amount of DNA inside a cell is assumed to be constant. However, the concentration of RNA is related to the amount of protein synthesis that is occurring. Hence, the ratio of RNA to DNA can be used as a reliable indicator of organism health and recent growth. Early work in fishes used a ultraviolet absorption procedure (Bulow, 1987). Low sensitivity (1.0 to 2.0 µg/ml) limited the application to relatively large amounts of tissue, necessitating the pooling of many larvae and masking individual variation that may be important. Improvement in the sensitivity and quantification of nucleic acids came with development of more sensitive fluorescent techniques. Several of these were based on the quantification of DNA using the fluorescent dye bisbenzimidazole (Hoechst 33258) and determining the concentration of total nucleic acids using ethidium bromide (Clemmesen, 1988) or thiazole orange (Berdalet and Dortch, 1989). The quantity of RNA was determined by the difference between the total nucleic acid fluorescence and DNA fluorescence. Other methodologies for nucleic acid concentration include the utilization of the nucleic acid enzymes RNase and DNase in combination with a non-specific fluorochrome such as ethidium bromide (Karsten and Wollenberger, 1977; Bentle et al., 1981; Dortch et al., 1983; Clemmesen, 1993; Westerman and Holt, 1994) or thiazole orange (Fara et al, 1996). In this technique, total nucleic acid concentration is determined on one portion of the sample while another portion of the sample is treated

with the nucleic acid enzyme, RNase, to digest RNA, resulting in the quantification of DNA alone. RNA again is calculated as the difference between total fluorescence and DNA fluorescence. DNase can then be added to the RNase treated sample to digest the remaining DNA, thus obtaining an estimate of the blank (Bentle et al, 1981; Westerman and Holt, 1994). However, McGurk and Kusser (1992) pointed out that the source of the fluorescence in the blank measured in this manner is of unknown cause. It may be due to the incomplete enzymatic digestion of RNA or DNA as well as compounds other than nucleic acids.

Each method described above has benefits and drawbacks associated with it. Methods using bisbenzimidazole have been reported to have poor reproducibility (Clemmesen, 1993; Fara et al., 1996) or require prolonged nucleic acid extraction techniques to improve the reproducibility (Clemmesen, 1988). Techniques using RNase and DNase can be time consuming due to the incubation time required.

The goal of this research was to develop a highly sensitive nucleic acid assay capable of accurately quantifying the RNA and DNA of individual gulf menhaden (*Brevoortia patronus*) and bay anchovy larvae (*Anchoa mitchilli*). Because this was one of three different biochemical measures of condition to be assessed (nucleic acid content, electron transport system activity and protein content), a further constraint was to make the assay compatible with the other two protocols. In addition to the work presented in this dissertation, the assay is being used in a laboratory based companion study on bay anchovy. Therefore, larvae as small as 2 day old anchovy (7 µg dry weight, E. Chesney, per. comm.) needed to be accurately measured for nucleic acid content, using as little of

the extracted material as possible so as to have sufficient tissue from each individual for all assays.

As a starting point, the methodology of Berdalet and Dortch (1991), which was originally developed for use with phytoplankton, was examined for potential modification. The technique uses bisbenzimidazole for DNA quantification and thiazole orange for total nucleic acid content determination of crude tissue homogenates. The authors choose to use thiazole orange instead of ethidium bromide because of a much greater sensitivity to both RNA and DNA. Thiazole orange had a 20-fold higher fluorescence yield when compared to ethidium bromide and also had relatively lower autofluorescence. This method is highly sensitive and would require small amounts of limited tissue homogenate for analysis. It was hoped that this technique could be quickly adapted for use on fish larvae. However, preliminary work confirmed the reports of poor reproducibility of the bisbenzimidazole. The technique described here is a combination of the dual fluorochrome methods of Berdalet and Dortch (1991) and the enzymatic digestion technique of Karsten and Wollenberger (1977). A technique similar to the one presented here has recently been published by Fara et al. (1996).

## **MATERIAL AND METHODS**

DNA type I from calf thymus, RNA from baker's yeast, Tris (Sigma 7-9) and DNase were purchased from Sigma (St. Louis, Missouri). RNase A from bovine pancreas and proteinase-K were purchased from U.S. Biochemical (Cleveland, Ohio). Thiazole orange was purchased through Aldrich Chemical Company.

To remove all organic contamination of glassware and plastic bottles, all containers were soaked in soapy water, rinsed with large amounts of tap water then cleaned with 10% HCl, then rinsed three times with distilled deionized water prior to use.

Concentrations of Thiazole orange (TO) and the molarity of Tris- $\text{Ca}^{++}$  buffer were followed from Berdalet and Dortch (1991). Stock solutions of TO (1 mg/ml) were made in ethanol. Stock RNA, stock DNA, and working solutions of RNA, DNA, TO, RNase, DNase and proteinase K were made in Tris buffer (0.1342 g  $\text{CaCl}_2 \cdot 7 \text{H}_2\text{O}$ , 12.11 g Tris(hydroxymethyl)aminomethane-Sigma 7-9®, 5.84 g NaCl, pH 7.6). Stock solutions of RNA and DNA consisted of approximately 5 mg of nucleic acid dissolved in 50 ml of freshly made Tris buffer; stock solutions were refrigerated and kept for extended periods (approximately 6 months). Working solutions of the nucleic acids were a 10:1 Tris dilution of the stock. The purity and concentration of the nucleic acid working solutions were checked daily measuring optical density at 230, 260, 275, 280 and 310 nm (Maniatis et al., 1982). Working solutions of TO were a 100:1 Tris dilution of the TO stock. All working solutions (including enzyme solutions) were made daily. All enzyme solutions were stored on ice.

The individual assays were comprised of 0.55 ml TO working solution plus Tris, enzyme solution (if needed), and nucleic acid standards (or larval extract) to equal 3.0 ml. The methods consisted of combining all components of the assay in a culture tube except the TO which was added while vortexing immediately prior to the fluorescence reading. All assay tubes were held in a room-temperature water bath to ensure that a

constant temperature was maintained throughout the series of trials. When a trial required two days to complete, water-bath temperature was noted on the first day and the water bath set accordingly the following day. All fluorescence readings, except where noted, were taken on a Turner Designs Model 430 spectrofluorometer (slit width 5 nm) with excitation and emission set at 511 and 533, respectively.

Gulf menhaden larvae used in the method development were collected at Port Fourchon, Louisiana during January, 1993. Larvae were collected using a light trap based on the quatrefoil design of Secor et al.(1991). Immediately after capture the larvae were placed small dish containing seawater from the collection site. The dish was placed into an ice bath to slow larval activity. Individual larvae were moved to a dissection microscope and photographed. The larvae were blotted dry and weighed to the nearest 0.1 mg using a Cahn Electro balance, rinsed in distilled deionized water, blotted dry, then frozen in liquid nitrogen.

Two methods were used to homogenize the larvae for nucleic acid extraction, grinding and sonication. For grinding, the larvae were removed from the liquid nitrogen and placed in a Potter-Elvehjem tissue homogenizing tube containing 2.0 ml Tris buffer and a 47 mm pre-combusted glass fiber filter. The larvae was ground for two minutes on ice. After grinding the homogenate was transferred to a conical centrifuge tube and the grinding tube and pestle were rinsed with 2.0 ml of Tris. For sonication, the larvae were removed from liquid nitrogen and placed into a small diameter (10x75 mm) culture tube containing 2.0 ml of Tris. The larvae were sonicated on ice at full power using a Fisher Model 50 Sonic Dismembrator equipped with a micro-tip probe. Sonication consisted of

7 bursts 5s in length, separated by 30 s cool down periods. Care was taken so that each larva was trapped perpendicularly between the probe tip and the bottom of the culture tube. For both techniques, the larval extract was stored on ice until needed.

A key component of this assay procedure is the additivity of the fluorescence from DNA and RNA. To test this property RNA standard curves were run with differing amounts of DNA standard added to the assay.

RNase and DNase digestion capabilities were examined by combining varied amounts of the enzymes to the nucleic acid standards. To determine the RNase concentration needed for the digestion of RNA and the effect of RNase on DNA, a series of nucleic acid standards (3.1 µg/assay RNA and 1.1 µg/assay DNA) were run with varying amounts of RNase (0, 10, 15 and 20 µg/3.0 ml assay) and incubated at 29° C for 30 min. A time series for optimal incubation was also conducted by incubating an additional set of assays containing 20 µg of RNase and nucleic acid standards for 30, 45, 60 and 75 min. Three replicates were run at each enzyme-nucleic acid-incubation time combination. After incubation the tubes were placed in a room temperature water bath for 2 min. The fluorescence was determined and compared with the fluorescence of a blank consisting of Tris, RNase and TO and standards containing Tris, nucleic acid standards (RNA or DNA) and TO.

A similar set of trials was completed for the examination of the digestive capabilities of DNase under the assay condition. A set of three replicates was set up containing varied amounts of DNase (30 and 60 µg/ 3.0 ml assay) and DNA (1.1 µg and 2.2 µg/ 3.0 ml assay). In an effort to increase the DNase activity, the cofactors  $Mg^{++}$  and

Mn<sup>++</sup> were added to a second trial at concentrations of 10 mM each to the 3.0 ml assay. After incubation at 29° C for 30 min the tubes were placed in a room-temperature water bath for 2 min. The fluorescence was determined and compared to a blank consisting of Tris, DNase and TO and standards containing Tris, DNA standards and TO.

Several studies involving the quantification of nucleic acids have indicated that greater nucleic acid extraction and detection occurs when the tissue homogenate is treated with protein enzymes. The effect of protease on the fluorescence intensity was examined by adding varied amounts of a mixture of 1 mg/ml proteinase-K solution to a cryovial containing Tris and larval extract. An individual menhaden larva was sonicated in 2.0 ml of Tris and 0.100 mL of the extract was distributed to cryovials containing varied volumes of the proteinase-K solution and Tris to equal 0.700 mL (0.800 mL total volume). The cryovials were incubated at 29° C for 30 min. After incubation, the cryovials were centrifuged (Eppendorf Centrifuge at 14,000 rpm) for 10 min. Three replicates were run at each protease concentration (0, 0.25, 0.375 and 0.625 µg/ ml). After incubation, 0.100 ml of the Tris-protease-larval extract combination was used in the fluorescence measurement. A time series of incubation time was conducted for 30, 60 and 90 min. with a protease concentration of 0.375 µg/ml.

It was desirable to be able to homogenize the larvae on one day and conduct the fluorescence portion of the nucleic acid assay the following day because the nucleic acid assay was to be used in combination with an electron transport system enzyme activity assay and a protein assay. To check the efficacy of this procedure, several larvae were sonicated individually and the total fluorescence from nucleic acid concentration

measured. After sonication, 0.100 mL was transferred to each of two cryovials containing 0.300 mL proteinase-K (1 mg/mL) and 0.400 mL Tris buffer. One of the cryovials for each larva was incubated and the total fluorescence read that day. The second cryovial was immediately frozen in liquid nitrogen. The following day, the second cryovial was placed in the water bath to incubate, then the total fluorescence was determined. The total fluorescence of the second day was compared to the total fluorescence of the first day. To control for the day to day variation in the spectrofluorometer sensitivity, the total fluorescence was normalized to the DNA standard curve each day before comparison, relying on the assumption that all the fluorescence of the two groups was due to DNA alone. A second trial was run identically to the above procedure, except that the specific amounts of DNA and RNA for each larvae were compared between fresh and frozen extract.

A comparison was made between the weight-specific DNA concentration of the two methods of homogenization to determine if sonication or grinding was more efficient at homogenizing the menhaden larvae. In the first comparison, the DNA concentration of larvae that had been ground was compared to the DNA concentration of sonicated larvae after both had been normalized to the wet weight of the individual larva. A second comparison was made by subjecting the homogenate of the sonication procedure to the standard grinding procedure then comparing the total fluorescence of the sonication-only homogenate to the total fluorescence of the sonication+grinding homogenate. If sonication does not completely homogenize the larva, the fluorescence of the sonication+grinding should be higher than sonication alone.



The sensitivity of the assay procedure was determined by finding the lowest nucleic acid concentration that resulted in fluorescence significantly greater than background autofluorescence. This is the only procedure of the assay-development portion of the study that was not conducted on the Turner Design Model 430 Spectrofluorometer. The fluorescence of this aspect of this part of assay development was determined on a Photon Technology International Quantum Master-1 spectrofluorometer.

To determine if nucleic acids were being lost during the assay procedure a series of 'spike' recovery experiments was performed. Immediately after sonication, a known amount of nucleic acid standard (spike) was added to a portion of the extract, an identical amount of Tris was added to a separate portion of the extract. If the assay procedure is reliable, the fluorescence (extract + 'spike') - fluorescence (extract alone) should equal the fluorescence of the spike alone. A total of 31 separate percent-recovery trials were run with 'spikes' ranging from 0.37 to 1.5  $\mu\text{g}$  DNA and 0.62 to 3.06  $\mu\text{g}$  RNA per 3.0 ml assay.

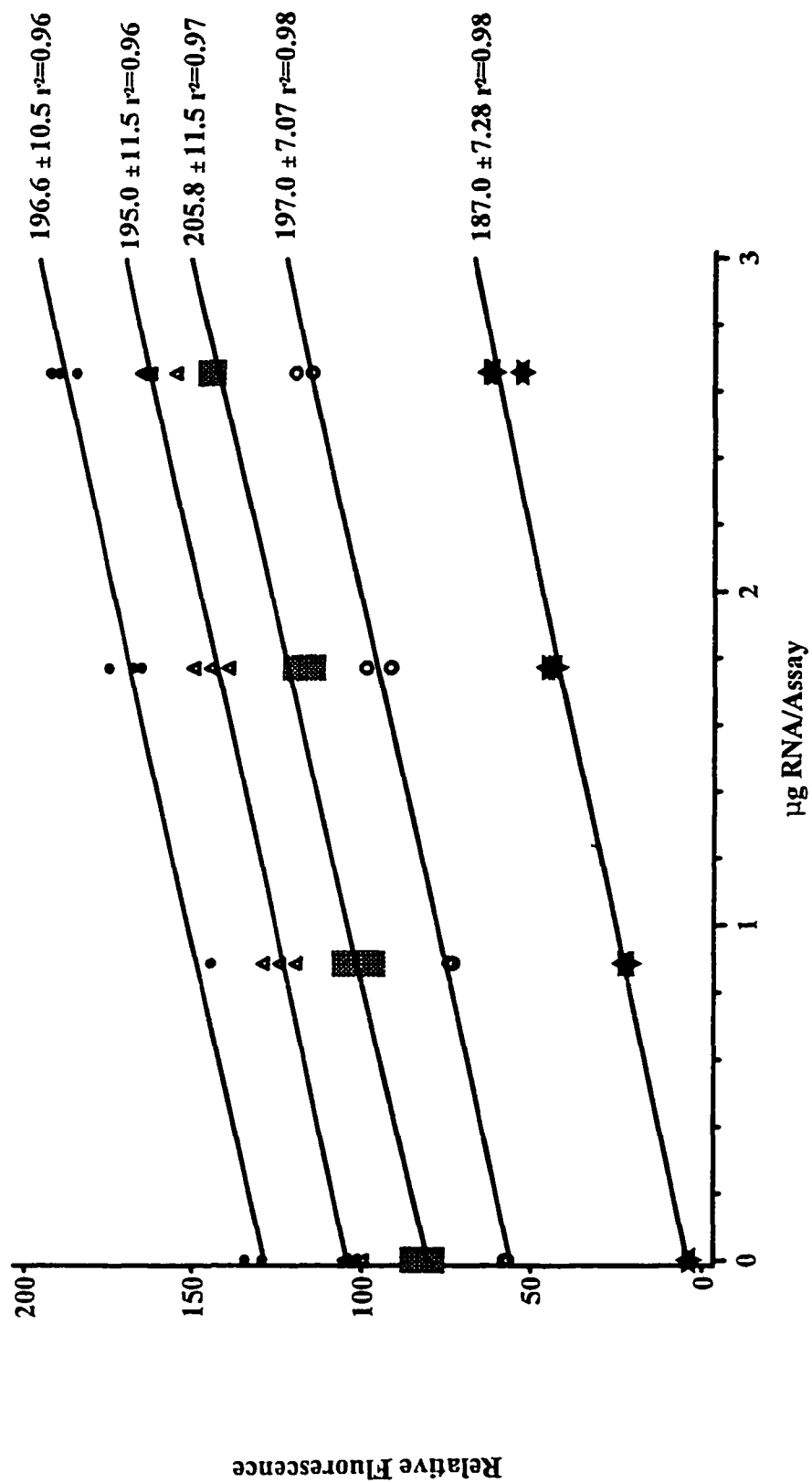
## **RESULTS**

### **Additivity of Fluorescence**

Thiazole orange showed strong fluorescence yield after binding with both DNA and RNA and proportionately low background fluorescence. For both nucleic acids the intensity of fluorescence was linearly related to nucleic acid concentration (Figure 2.1).

**Figure 2.1: Results of the check of additivity of the fluorescence of RNA and DNA. RNA standard curves with varied amounts of DNA standard added. ★ 0.0  $\mu\text{g}$  DNA/assay, ○ 0.067  $\mu\text{g}$  DNA/assay, ■ 1.0  $\mu\text{g}$  DNA/assay, △ 1.33  $\mu\text{g}$  DNA/assay, • 1.67  $\mu\text{g}$  DNA/assay. Slope, standard error of the slope and  $R^2$  for each regression are listed at the end of each regression line.**

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Analysis of covariance of RNA standard curves showed that the linear increase in fluorescence was independent of DNA concentration. The slope of the RNA regression standards was not influenced by the presence of DNA ( $p=0.72$ ), and the fluorescence of RNA and DNA was additive .

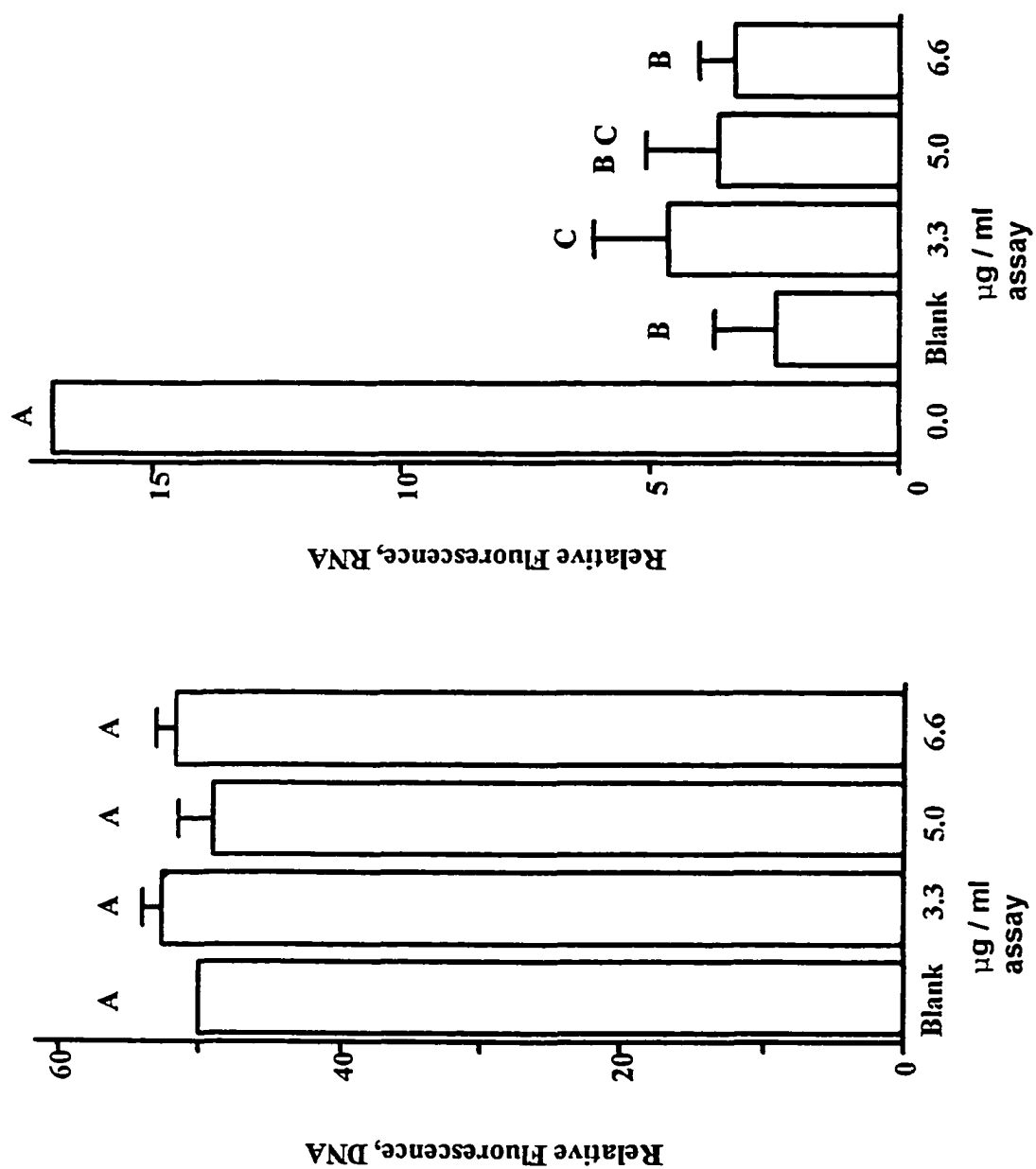
### **RNase Digestion of RNA and DNA**

RNase concentrations of 20  $\mu\text{g}/\text{assay}$  (6.67  $\mu\text{g}/\text{ml}$ ) incubated for 30 min at 29° C were sufficient to eliminate RNA standards present in the assay (Figure 2.2). The fluorescence of >3  $\mu\text{g}/\text{assay}$  of RNA was reduced by 98% with the addition of 20  $\mu\text{g}$  of RNase and was not significantly different than the autofluorescence of the dye alone ( $p>0.3$ ). Increases in incubation time had no effect on the amount of reduction of fluorescence (data not shown). There was no effect of RNase on the fluorescence of DNA. DNA incubated in the presence of 20  $\mu\text{g}/\text{ml}$  of RNase had fluorescence equivalent to that of equal amount of DNA alone.

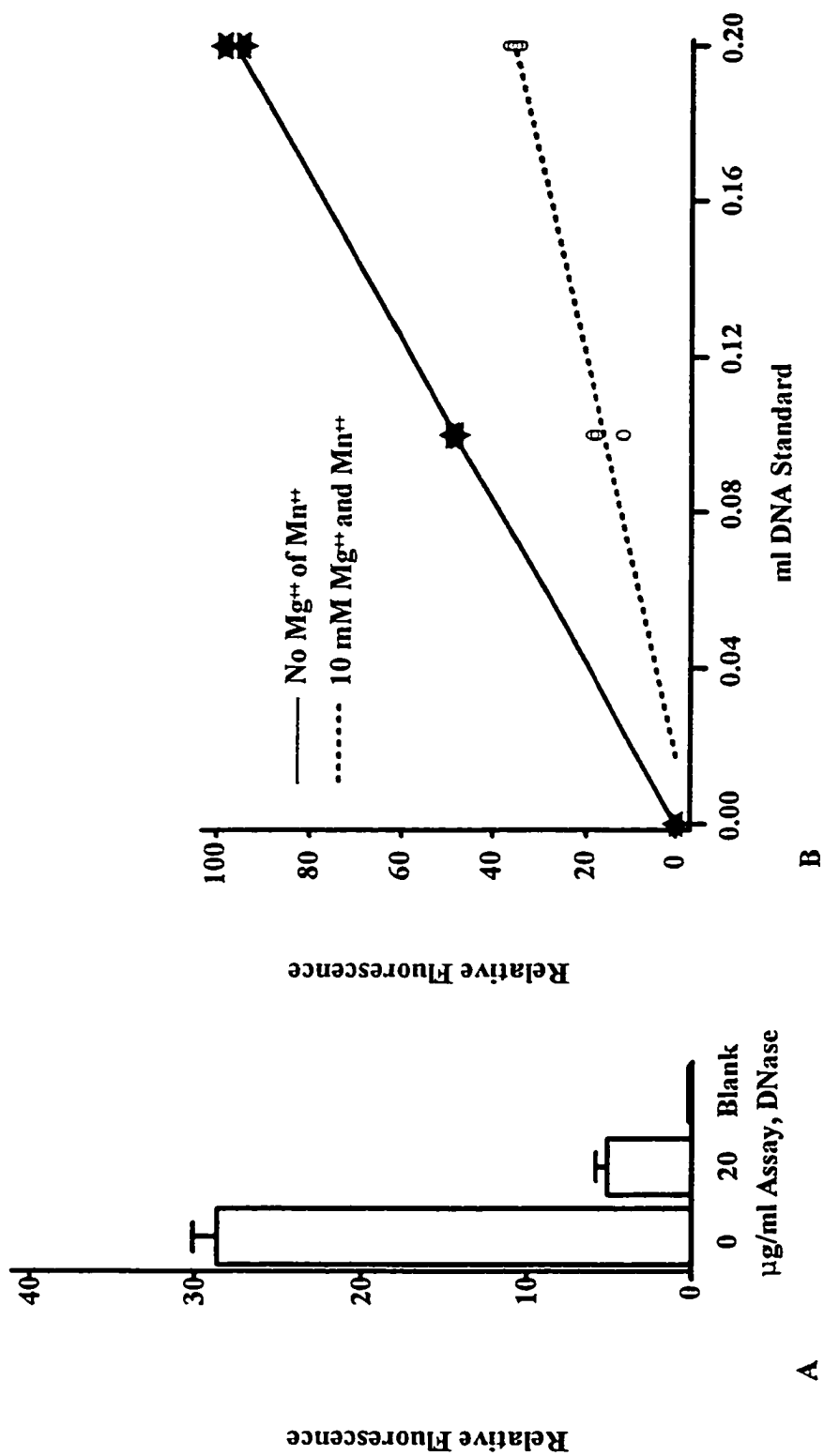
### **DNase Digestion of DNA**

DNase in the standard Tris buffer was not sufficient to remove the fluorescence of DNA standards to blank levels (Figure 2.3a). The addition of the divalent cations,  $\text{Mg}^{++}$  and  $\text{Mn}^{++}$ , increased the digestive capabilities of the DNase, but fluorescence levels were still greater than the blank. Concurrent with the addition of the divalent cations to the assay, there was a 37% decrease in the fluorescence of the DNA standards (Figure 2.3b). The failure of the DNase to remove DNA standards below the detectable limits of this assay and the reduction of fluorescence with the addition of divalent cofactors precluded the use of DNase .

**Figure 2.2: RNase digestion of DNA (a) and RNA (b). Bars represent different amounts of RNase added to 3.0 ml assay. Blank is 0 RNase, 0 RNA standard. DNA standard concentration=0.66  $\mu\text{g}/\text{assay}$ , RNA standard concentration=0.74  $\mu\text{g}/\text{assay}$ . Incubation 30 min., at 29° C. Error bars represent 1 standard error. Means with the same letter are not significantly different (Tukey multiple comparison,  $\alpha = 0.05$ ).**



**Figure 2.3: a) DNase digestion of DNA. Blank 0 DNase, 0 DNA standard. DNA standard concentration=0.60  $\mu\text{g}/\text{assay}$ . Incubation 30 min.,. at 29° C. b) Reduction in the fluorescence signal with the addition of 10 mM  $\text{Mg}^{++}$  and 10 mM  $\text{Mn}^{++}$ .**





## **Protease**

The addition of a protease to the homogenate was needed to eliminate the activity of the naturally occurring RNase present in the larvae and has been reported in the literature to increase sample fluorescence. The addition of the proteinase K also increased the overall fluorescence of the homogenate by up to 100%, presumably by removing nucleoproteins which may have hindered the binding of Thiazole orange. The maximum increase in total fluorescence resulted from the addition of 300  $\mu$ l of a 1 mg/ml Proteinase K solution to 100  $\mu$ l homogenate diluted in 400  $\mu$ l of Tris. The addition of greater concentrations of Proteinase K had no effect on the fluorescence of the sample (Figure 2.4a). Incubation times of 30 min were sufficient for maximum fluorescence and additional time had no effect on fluorescence (Figure 2.4b).

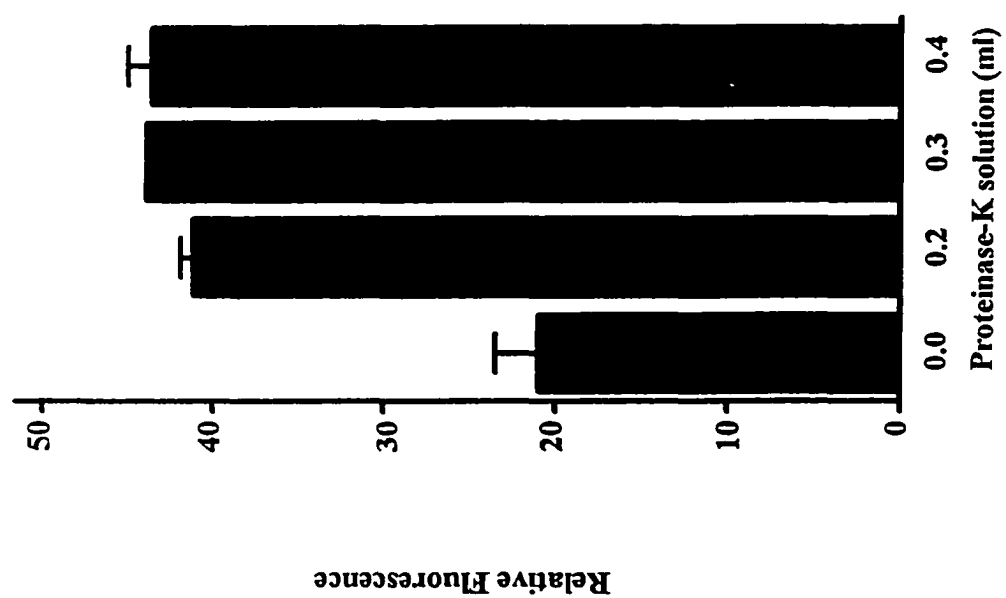
## **Freezing of Extract**

Fast freezing the larval homogenate in liquid nitrogen overnight resulted in an increase in the total fluorescence of the extract and apparent DNA concentration with no change in the RNA measurements. Total fluorescence, normalized to daily standard curves, increased by 36.8% (se=10.9, n=4). The measurement of DNA increased by a mean of 14.7% , there was no significant change in RNA but the measurement decreased by 4% after freezing (Table 2.1). Longer periods (48 h) of freezing resulted in highly variable results with no consistent trends in either RNA or DNA concentrations.

## **Sonication vs Grinding**

There was a difference between grinding and sonication for the extraction of DNA. Sonication gave a higher DNA-to-wet-weight ratio than grinding ( $p=0.04$ ). Also,

**Figure 2.4: Proteinase-K digestion of 0.10 ml larval extract. Proteinase-K concentration was 1.0 mg/ml. Tris was added to each volume of Proteinase-K solution to make a total volume of 0.7 ml. Incubation was 30 min at 29° C. Error bars represent 1 standard error.**



**Table 2.1: Nucleic acid content of two menhaden larvae. Comparison of fresh and frozen extract. Presented are the means of three replicates for each larva. There is a significant increase in DNA content after freezing (Randomized block ANOVA,  $\alpha=0.05$ ).**

	<b>Fresh DNA (<math>\mu\text{g}/\text{assay}</math>)</b>	<b>Frozen DNA (<math>\mu\text{g}/\text{assay}</math>)</b>	<b>Fresh RNA (<math>\mu\text{g}/\text{assay}</math>)</b>	<b>Frozen RNA (<math>\mu\text{g}/\text{assay}</math>)</b>
<b>Larvae A</b>	0.410	0.485	1.45	1.48
<b>Larvae B</b>	0.431	0.482	1.34	1.42

the grinding of the larval homogenate after the sonication showed no increase in the fluorescence of the sample (Table 2.2).

### **Sensitivity of Thiazole Orange**

The lower limit of detection for the of DNA standards was 10 ng per assay (3.33 ng/ml). The fluorescence at this concentration was significantly greater than the blank ( $p < 0.001$ ). RNA standards were detectable to 47 ng/assay (15.67 ng/ml,  $p < 0.001$ ).

### **Percent Recovery of Nucleic Acids**

A total of 31 separate spike recovery trials were conducted. For the entire data set the mean recovery was 102.4 % (SD=0.058) and 92.6% (SD=0.16) for DNA and RNA, respectively. It was apparent from the examination of Figure 2.5 that the recovery of both nucleic acids deviated from 100% as the total nucleic acid concentration of the assay increased, probably as a result of the failure of the RNase to completely digest the RNA, resulting in a greater than 100% recovery of the DNA and a lower than 100% recovery of the RNA. Based on Figure 2.5, an upper limit of total nucleic acid spike concentration was set at 3 µg/assay and the percent recovery calculations were re-computed. The recoveries at lower nucleic acid concentrations were closer to the 100% optimum and additionally had lower deviation,  $\bar{x}$  DNA=100.2 % (SD=0.038) and  $\bar{x}$  RNA= 99.6% (SD=0.92). Since the assay was sensitive to levels well below 1 µg for both nucleic acids, all analyses were kept below the 3 µg upper limit.

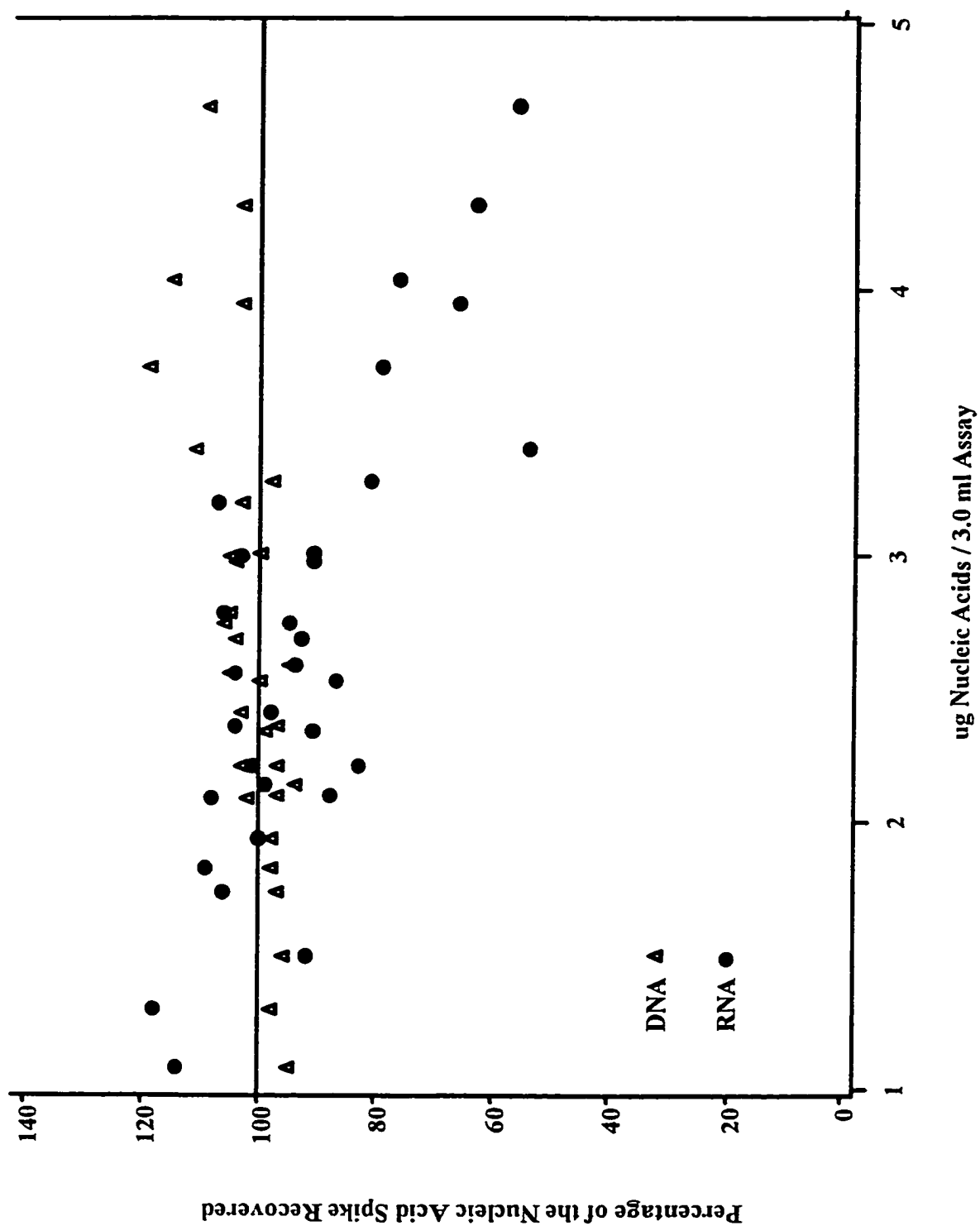
## **DISCUSSION**

Based on the results, the following procedure was chosen for the extraction and quantification of nucleic acids of larval menhaden. The larvae are sonicated on ice in 2.0

**Table 2.2: Comparison of sonication and grinding as methods of tissue homogenization. Top half of the table are the results of the wet weight specific DNA of sonicated larvae (n=10) and ground larvae (n=7). DNA values are  $\mu\text{g}/3.0$  ml assay. The lower half of the table shows no increase in relative fluorescence ( $\bar{x} \pm \text{standard error}$ ) occurs after grinding sonicated tissue (n=3).**

	<b>Sonication</b>	<b>Grinding</b>
<b>DNA/Wet weight</b>	1.609	1.306
	<b>Sonication Only</b>	<b>Sonication + Grinding</b>
<b>Total Fluorescence</b>	83.67 $\pm$ 0.88	84.0 $\pm$ 0.58

**Figure 2.5: Percent recovery of nucleic acid standard spikes. A reference line is drawn at 100% recovery for comparison.**





ml of Tris and 0.100 ml of the resulting homogenate is combined with 0.400 ml Tris and 0.300 ml of proteinase-K (1 mg/ml). The solution is stored overnight in liquid nitrogen. After being removed from the nitrogen, the extract is incubated at 29° C for 30 min. The extract is then centrifuged for 10 min to remove cellular debris. To measure total nucleic acid concentration, 0.05 ml of the extract is combined with 2.40 ml Tris and 0.55 ml Thiazole orange is added while the mixture is vortexed. The fluorescence is read with excitation wavelength of 511 nm and emission wavelength of 533 nm. To measure DNA fluorescence, 0.05 ml of the extract is combined with 2.20 ml Tris and 0.200 ml RNase (0.3 mg/ml) and allowed to incubate at 29° C for 30 min. The mixture is then moved to a room temperature water bath to cool for 2 min. Thiazole orange is added (0.55 ml) and the fluorescence is determined. The calculations for RNA and DNA content of individual fish larvae are:

$$DNA (\mu g/larvae) = \frac{DNA_{FL} - DNA_{Intercept}}{DNA_{slope}} \times \frac{Cryo. Vol}{DNA Ext.} \times \frac{Son. Vol}{NA Ext.}$$

$$RNA (\mu g/larvae) = \frac{Total_{FL} - DNA_{FL} - RNA_{Intercept}}{RNA_{Slope}} \times \frac{Cryo. Vol.}{Total Ext.} \times \frac{Son. Vol.}{NA Ext.}$$

Where  $DNA_{FL}$  and  $Total_{FL}$  are the fluorescence readings for the DNA and total nucleic acid assay,  $DNA_{Slope}$  and  $DNA_{Intercept}$  are the slope and intercept of the DNA standard curve. Those for the RNA standard curve are similarly subscripted. Cryo. Vol. is the volume of the protease, larval extract and Tris that was stored frozen (0.800 ml). DNA and Total extract are the amounts of the cryo vol. used in the assay (0.05 ml). Son. Vol.

is the sonication volume (2.0 ml) and NA ext is the amount of sonication volume set aside for the nucleic acid assay (0.100 ml).

The strong linear relationship between nucleic acid concentration and the magnitude of fluorescence indicates that thiazole orange is an appropriate fluorochrome for the quantification of RNA and DNA. In general, the standard curves were very consistent and the failure to obtain a  $r^2$  approaching 1.0 could be used as an indicator of problems associated with the cleanliness of glassware and purity of the reagents. The additivity of the fluorescence of RNA and DNA confirm that the mathematical protocol for the determination of RNA fluorescence (total fluorescence-DNA fluorescence) is appropriate. The additivity of the fluorescence seems to be promoted by the presence and concentration of the  $\text{Ca}^{+}$  in the Tris buffer (Dortch, pers. comm). Additivity curves run with differing concentrations of  $\text{Ca}^{+}$  (in an attempt to promote DNase activity, Bentle et al. , 1981) proved non-parallel, though still strongly linear. The sensitivity of 3.3 ng/ml for DNA and 15.67 ng/ml for RNA are lower than most studies using ethidium bromide (Caldarone and Buckley, 1991; Clemmesen, 1993; Canino and Caldarone 1995) and similar to the thiazole orange methods (Berdalet and Dortch, 1991; Fara et al., 1996). It should be noted that this was the only portion of the protocol development that was investigated on a new instrument. However, the general performance of the Turner Design spectrofluorometer (the old instrument) and of the Photon Technologies International spectrofluorometer were very similar. The limit of detection using thiazole orange is limited by the self fluorescing blank rather than the ability of the machine to detect low levels of fluorescence.

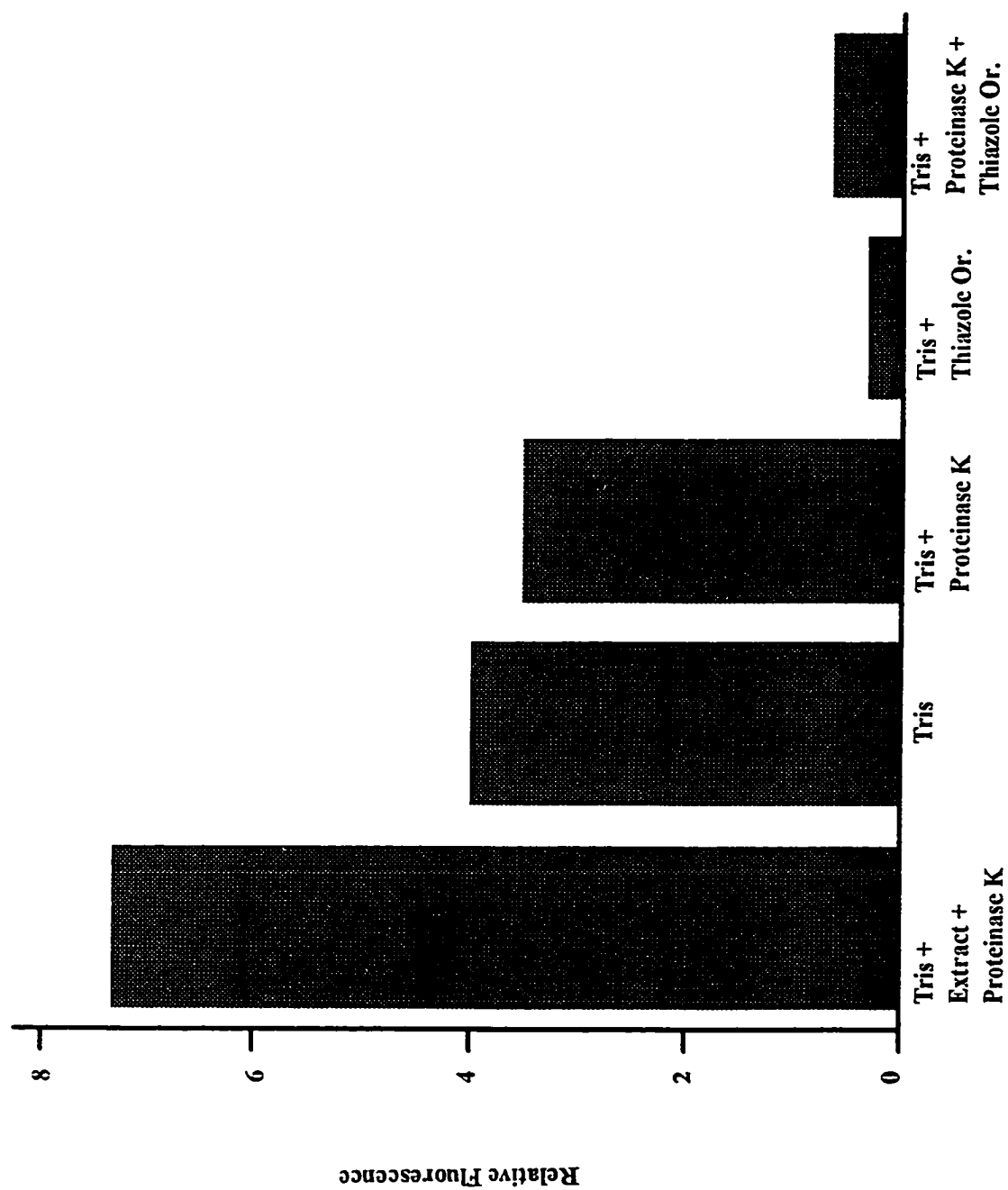
The concentration of RNase needed for the complete digestion of the RNA standards was 20  $\mu\text{g}$  per 3.0 ml assay (6.67  $\mu\text{g}/\text{ml}$ ) incubated at 29° C for 30 min. This concentration is similar to Clemmesen (1993) in which 10  $\mu\text{g}/\text{ml}$  was used and resulted in residual fluorescence equivalent to the blank after incubation. This was the lowest concentration examined by Clemmesen (1993). Bentle et al. (1981) used RNase concentrations of 25  $\mu\text{g}/\text{ml}$  and Karsten and Wollenberger (1977) had final RNase concentrations of 50  $\mu\text{g}/\text{ml}$ . Using only 0.5  $\mu\text{g}/\text{ml}$  RNase, Fara et al. (1996) obtained a residual fluorescence of 10% of the untreated RNA standard and the authors reported the residual fluorescence was independent of RNase concentrations up to 100  $\mu\text{g}/\text{ml}$ . In this study 6.67  $\mu\text{g}/\text{ml}$  RNase resulted in fluorescence indistinguishable from the blank and RNase level of 3.3  $\mu\text{g}/\text{ml}$  did not fully digest the RNA. RNase concentrations required for the complete digestion of RNA in this study had no effect on the fluorescence of DNA. Clemmesen (1993) found that RNase concentrations greater than 50  $\mu\text{g}/\text{ml}$  lead to a decrease in DNA fluorescence which the author attributed to RNase digestion of the DNA. RNase digestion of DNA was not observed in this study, but the concentrations used were much lower than those of Clemmesen (1993). The wide range of RNase concentrations reported in the literature could be taken as evidence that there is great variability in the purity and activity of the commercially available enzymes. The only method to ensure complete (or satisfactory) digestion of RNA is to test a variety of concentrations under the specific assay conditions at hand.

The ability of DNase to digest DNA in this assay procedure was unsatisfactory. The fluorescence remaining after incubation of DNA standards with as much as 20  $\mu\text{g}/\text{ml}$

was significantly greater than the autofluorescence of thiazole orange. The addition of the cofactors  $\text{Mn}^{++}$  and  $\text{Mg}^{++}$ , increased the digestion of the DNA but reduced the fluorescence signal of the nucleic acids. Since this would result in a loss of sensitivity, it was deemed unacceptable. The remaining fluorescence after digestion of 0.54  $\mu\text{g}$  of DNA was equivalent to the fluorescence of approximately 60 ng of DNA standard. This is well above the detection limit (10 ng/3.0 ml assay) of this assay but below some published ethidium bromide techniques (Clemmesen, 1988; Bentle et al., 1981). Thus, while DNA digestion may appear to be “complete” in an assay utilizing ethidium bromide, it cannot serve as an estimator of the blank in assays using thiazole orange. In their thiazole orange based estimate of RNA content using DNase, Fara et al. (1996) use a 20% correction to adjust for residual DNA fluorescence after DNase incubation. Caldarone and Buckley (1991) presented a flow injection analysis methodology using Hoechst 33258 and ethidium bromide and reported DNA detection limits of 10 ng. They also report that the DNase digestion may not have been complete, resulting in high residual fluorescence.

Because the failure of DNase to completely remove all of the DNA standards present in the assay, determination of the appropriate blank for the larval extract became difficult. There needed to be a control for compounds within the extract that may produce self-fluorescence. The fluorescence of several combinations of Tris, extract, proteinase-K and dye are shown in Figure 2.6. The largest blanks appear when the dye

**Figure 2.6: Blank levels for different combinations of assay constituents.**



is not present. The addition of the dye reduced the “fluorescence” of Tris alone, and Tris+Proteinase-K by over 80%. The fluorescence values of Tris alone and Tris+proteinase-K may be due to the scattering of the excitation light, which then impinges upon the emission receptor. The excitation and emission wavelengths of thiazole orange are separated only by 22 nm, where ethidium bromide and Hoechst 33258 are separated by 225 nm and 100 nm, respectively. It seems possible that scattered 511 nm light may invoke a false reading of an emission receptor set for 533 nm. The addition of thiazole orange would help to absorb this scattered light. The blank associated with the Tris+proteinase-K+extract was highest. But this combination would also have the scattered light contributing to the magnitude of the blank. This blank was also greater than the residual fluorescence remaining after the incomplete DNase digestion of the RNase treated extract, therefore whatever is contributing to the self-fluorescence of the Tris+proteinase-K+extract is suppressed when the dye is added. Based on these results, it was concluded that the extract of menhaden larvae contained no self-fluorescent blank, which is reasonable given that the larvae are only lightly pigmented and pigments contribute to the magnitude of the blank. Other RNA-DNA methodological studies using fish larvae have found little or no self-fluorescent blank (Caldarone and Buckley, 1991; Clemmesen, 1993; Canino and Caldarone 1995).

The addition of proteinase-K and the fast freezing of the extract both increased the fluorescence signal of the larval extract. Proteinase-K probably removes the nucleoproteins and makes the DNA more accessible to thiazole orange. Similar increases were reported by Karsten and Wollenberger (1977), Bentle et al. (1981) and

Clemmesen (1988). Berdalet and Dortch (1991) reported that protease addition only slightly increased the recovery of DNA in phytoplankton while decreasing the RNA recovery. This is perhaps a result of RNase contamination of the protease that was reported by Karsten and Wollenberger (1977). The proteinase-K used here had no effect on the fluorescence of the RNA or DNA standards and is unlikely to have RNase contamination. Fast freezing was reported to increase the recovery in marine bacteria (Fara et al., 1996). No explanation is stated but the increases they observe and the increases reported here may be a result of the disruptions of coagulations of proteins, cell debris and nucleic acids by the ice crystals making these constituents more accessible to the protease and the nucleic acids more accessible to the fluorochrome.

Sonication was a superior method for the extraction of nucleic acid.

Procedurally, sonication was much easier when care was taken to ensure that complete disruption occurred with no loss of sample or sonication intensity by the foaming of the extract. With larger larvae (15-20 mm TL), it was necessary to trap the larvae under the sonication probe tip. This allowed the full force of the sonication to be directed at the larvae. A microscopic examination of the homogenate after sonication revealed the presence of only bony material (skull, vertebra and otoliths).

The complete test of an extraction procedure is the recovery of RNA and DNA standards that are added as 'spikes' to the extract. If the method is sound there should be no loss of either nucleic acid. The method of enzymatic digestion of RNA in combination with the fluorochrome thiazole orange described in this study produced



recoveries 100.2% and 99.6% for DNA and RNA respectively, for assays with total nucleic acid concentration below 3.0 µg/assay.

The method developed with this study is fully compatible with the assays for the determination of ETS activity and protein content. The ability to fast freeze larval homogenate after sonication without loss of nuclear material provides an increased amount of time to accomplish multiple assays. The assay is highly sensitive and has been used to quantify the nucleic acid content of individual 2 day-old anchovy larvae using only 30% of the total homogenate volume. Using sonication to extract the nucleic acid also allows for otolith recovery, which can provide additional information to larval fish studies (although not determined, it is likely that otoliths are lost in grinding).

## **CHAPTER 3: BIOCHEMICAL MEASURES OF CONDITION**

### **INTRODUCTION**

Marine fish populations undergo dramatic fluctuations in year-class strength, and it is thought that the cause of these fluctuations is variations in larval survival. In a simple demonstration of this process, Houde (1989) showed that changes as small as 5% day<sup>-1</sup> in the growth or mortality rates of marine fish larvae can produce an order of magnitude difference in the number of individuals surviving through the larval stage. Generally, the variance associated with the estimates of growth and mortality exceeds this 5% day<sup>-1</sup> level. As a result, there has been a need to better quantify the relative health or condition of marine fish larvae to more accurately predict the variables that influence year-class strength.

The nutritional state of a larva has a major role in its survival because of its effect on the larva's ability to avoid predators (Bailey and Houde, 1989), maintain vertical position in water column (Sclafani et al., 1993), and larval stage duration (Ferron and Leggett, 1994). Various biochemical and morphological condition indices have been developed in an effort to quantify the relative health of marine fish larvae. Among these indices of condition are morphometric analysis, histological analysis, the ratio of RNA to DNA content, metabolic and digestive enzyme activity, and protein content. Laboratory calibration has shown each of these methods to represent the recent feeding history of the individual larvae to varying degrees. Morphometric analysis of condition generally consists of computing a ratio of body measurements affected by low food densities (e.g. body depth) to body characteristics thought to be "starvation resistant" (e.g. eye

diameter). Histological analyses of liver, pancreatic and gut tissues has shown that the cell characteristics can be used to accurately predict the recent feeding success in the laboratory as well as in field caught specimens (Theilacker, 1986).

The biochemical indices (RNA:DNA, enzyme activity and lipid and protein concentration) all have been well characterized in laboratory settings. RNA:DNA values are based on the fact that DNA levels are constant within a cell, but RNA concentration will be in direct relation to the amount of protein synthesis, hence growth, occurring within the cell. A large number of methodological and calibration studies have shown that RNA:DNA values reflect the recent feeding success of marine fish larvae (Clemmesen, 1988, 1989; Westerman and Holt, 1994). Electron transport system (ETS) enzymes have been used to estimate the potential respiration rates in marine plankton (Packard, 1985) and bacteria (Packard et al., 1983). More recently, ETS activity has been measured in fish eggs and larvae (Yamashita and Bailey, 1990; Pfeifer and Govoni, 1993; G.-Toth et al., 1995) and marine invertebrates (Savenkoff et al. 1995; Savenkoff et al. 1995). ETS activity is used as a proxy measure for respiration, and therefore is an indicator of the metabolic rate of the organism. Other metabolic enzymes shown to be correlated with recent feeding in fish larvae and invertebrates include lactate dehydrogenase (Clarke et al., 1992) and citrate synthase (Clarke et al., 1992; Clarke and Walsh, 1993). Since fish can metabolize muscle tissue during periods of starvation, protein concentration can also be used as an indicator of larval condition. In laboratory studies, the ratio of RNA to protein can serve as indicators of protein production and growth (Foster et al., 1993; Mathers et al., 1993). In addition to representing recent

growth, protein concentration can affect the buoyancy and vertical distribution of larvae by altering the water content of the body tissues. Lipids are the storage product and energy reserves of fish and are utilized during times of starvation in adult and juvenile fish. However, the low levels found in larvae and the conflicting data in the literature led Ferron and Leggett (1994) to conclude that lipid analysis has limited application in quantifying the condition of larval fish. With the refinement of techniques, these biochemical indices are being utilized in the field to examine how larval growth is affected by environmental and anthropogenic influences (Koslow et al., 1985; Robinson and Ware, 1988; Powell et al., 1988; Burke et al., 1993; Foster et al., 1993; Rooker and Holt, 1997).

The gulf menhaden (*Brevoortia patronus*) is of particular interest along the Gulf coast of the United States. The menhaden fishery is the largest (by weight) in the continental United States (Lassuy, 1983), and all of the life stages of menhaden serve as an important forage for many commercially and recreationally important species. Gulf menhaden are estuarine dependent: the adults spawn in the near shore area off the coast of Louisiana in the fall and winter (Shaw et al., 1985a) and the larvae are transported into the estuaries via episodic wind events that result from the progression of atmospheric cold fronts (Shaw et al., 1985b; Leach, 1994). Cold-front passage occurs along the Louisiana coast during the winter months with a frequency of 3-8 d (Ward, 1980). As the low-pressure system moves south or southeast across the great plains, the resultant southerly winds increase and push Gulf of Mexico water into the estuaries. As the front moves past the coastline, the wind shifts quickly to the north to push water out

the estuarine passes (Ward, 1980). The purpose of this study was to quantify the condition of the menhaden larvae as they enter the Louisiana estuarine system, and determine what effect, if any, the increased winds associated with these frontal passages might have on the condition of the larvae.

Atlantic menhaden (*B. tyrannus*) were shown to have decreased growth, based on otolith increment analysis, in association with the winds of winter storms off the coast of North Carolina (Maillet and Checkley, 1991). Results like those of Maillet and Checkley (1991) tend to support the stable-ocean hypothesis (Lasker, 1975). Strong winds causing vertical mixing tend to disrupt aggregations of larval prey items, effectively reducing prey concentration. However, Dagg (1988) suggested that the passage of a cold front along the coast of Louisiana will increase production of copepod nauplii. It was hypothesized that the redistribution of nutrient-rich, near-shore water to the more depleted offshore region would stimulate offshore production. The resultant increase in offshore productivity would then quickly stimulate egg production in copepods, thus increasing the concentration of food for larval fish.

More recent work has suggested that the generation of turbulence will increase the encounter rate of larvae and their prey, leading to increased feeding. MacKenzie et al. (1994) present a model that predicts a domed shape response of larval fish ingestion rates in relation to turbulence. Building on an encounter rate model from Rothschild and Osborn (1988) and incorporating predator reactive distance and pursuit time, MacKenzie et al. (1994) state that larval fish ingestion rates should increase with increasing turbulent velocity until the probability of successful pursuit decreases due to the prey

advection beyond the predator reactive distance. Davis et al. (1991) incorporate plankton patchiness in their turbulence model and predict that the turbulence associated with light to moderate winds ( $\sim 5 \text{ m s}^{-1}$ ) will decrease feeding and growth by the destruction of food patches. At wind speed of  $\sim 10 \text{ m s}^{-1}$ , increased encounter rates overcome the detrimental effects of patch dispersion. MacKenzie et al. (1994) predict that maximum ingestion rates will occur at wind speeds of  $\sim 15\text{-}17 \text{ m s}^{-1}$ . Sundby et al. (1994) found a 7-fold increase in the feeding of cod larvae (*Gadus morhua*) when winds increased from 2 to  $10 \text{ m s}^{-1}$ . Wind speeds associated with cold-front progression in south Louisiana are typically  $3\text{-}7 \text{ m s}^{-1}$ .

The objective of this study was to examine the influence of atmospheric cold-front passage on the condition of menhaden larvae. A series of three biochemical assays, nucleic acid concentration, protein content and ETS activity were conducted on individual larvae in the effort to detect and quantify any change in their condition. From these three biochemical measures, a series of nine condition indices were generated (Table 3.1) and correlated to each other and to daily atmospheric conditions. In addition to the overall effect of frontal passage, the inter-relationship of all the biochemical indices was explored. Finally, the level of larval condition in relation to the progression of the spawning and winter season was explored.

## **MATERIAL AND METHODS**

### **Larval Collection**

The study site was located on a tidal creek at Port Fourchon, LA approximately 3.3 km inland of Belle Pass and Pass Fourchon. The site is representative of most

**Table 3.1: Biochemical measures of condition used for this study.**

<b>Biochemical Measure</b>	<b>Measure of size or Condition</b>	<b>Reasoning</b>	<b>Selected References</b>
RNA	Condition	RNA concentration reflects levels of protein synthesis	Clemmesen, 1988;
DNA	Size	Estimate of the number of cells in the larvae	
RNA:DNA	Condition	DNA is constant, while RNA varies with protein synthesis	Clemmesen, 1988; Buckley, 1987; Westerman and Holt, 1994
ETS	Condition	Measure of potential respiration	Packard, 1985; Yamashita and Bailey, 1991
ETS Protein <sup>-1</sup>	Condition	Size-corrected respiration potential	
ETS DNA <sup>-1</sup>	Condition	Size-corrected respiration potential, per-cell estimate of respiration	Savenkoff et al., 1995
Protein	Both	Fish are up to 50% by weight protein and will catabolize muscle tissue during starvation	Richard et al., 1991
RNA Protein <sup>-1</sup>	Condition	RNA efficiency, measure of the rate which proteins are produced	Foster et al., 1993; Mathers et al., 1993
Protein DNA <sup>-1</sup>	Condition	Per-cell estimate of protein concentration	

Louisiana estuarine systems; the area is subjected to low tidal range ( $\sim 0.3$  m) and the circulation and water movement is meteorologically dominated (Ward, 1980). Daily collections of menhaden larvae were made during 2 week sampling trips in December, January and February of 1993-1994 and 1994-1995. Collections were made using a light trap (Secor et al., 1991) set in the surface waters immediately after sunset. Typical deployment times were 15 min. Upon retrieval of the trap, the cod end was immediately removed and the contents gently poured into a bucket containing 15 L of seawater from the collection site. The larvae were moved into the lab and a cursory count was taken. If fewer than 30 larvae had been captured, the trap was re-deployed until 30 larvae were captured or it became apparent that the larvae were not present or were unavailable to the trap due to water clarity. After all the larvae had been collected for the day, the larvae were transferred in small groups (7-10 individuals) from the bucket to a small dish, which was then immersed into an ice bath. After approximately 5 min in the ice bath, the larval movement slowed enough that the larva could be photographed. Individual larvae were gently grasped with delicate forceps and placed on a small piece of glass immersed in chilled seawater. The piece of glass had "V" shaped grooves that consistently positioned the larva prior to the photography. The larva was photographed, given a brief rinse with distilled dionized water (DDW), blotted dry, placed in a cryovial and immediately frozen in liquid nitrogen. Care was taken so that all the larvae used for condition measures had survived the chilling and photographic manipulations. Chilling the larva was a necessary step before acceptable photographs could be taken. The effect of chilling on the biochemical measures of condition was tested by freezing a series of



larvae directly from the 15 L bucket (after a DDW rinse and blot dry) and larvae that had been taken from the same collection but chilled prior to freezing.

### **Assay Protocol**

A complete summary of the extraction technique and the protocol of each assay is shown Figure 3.1.

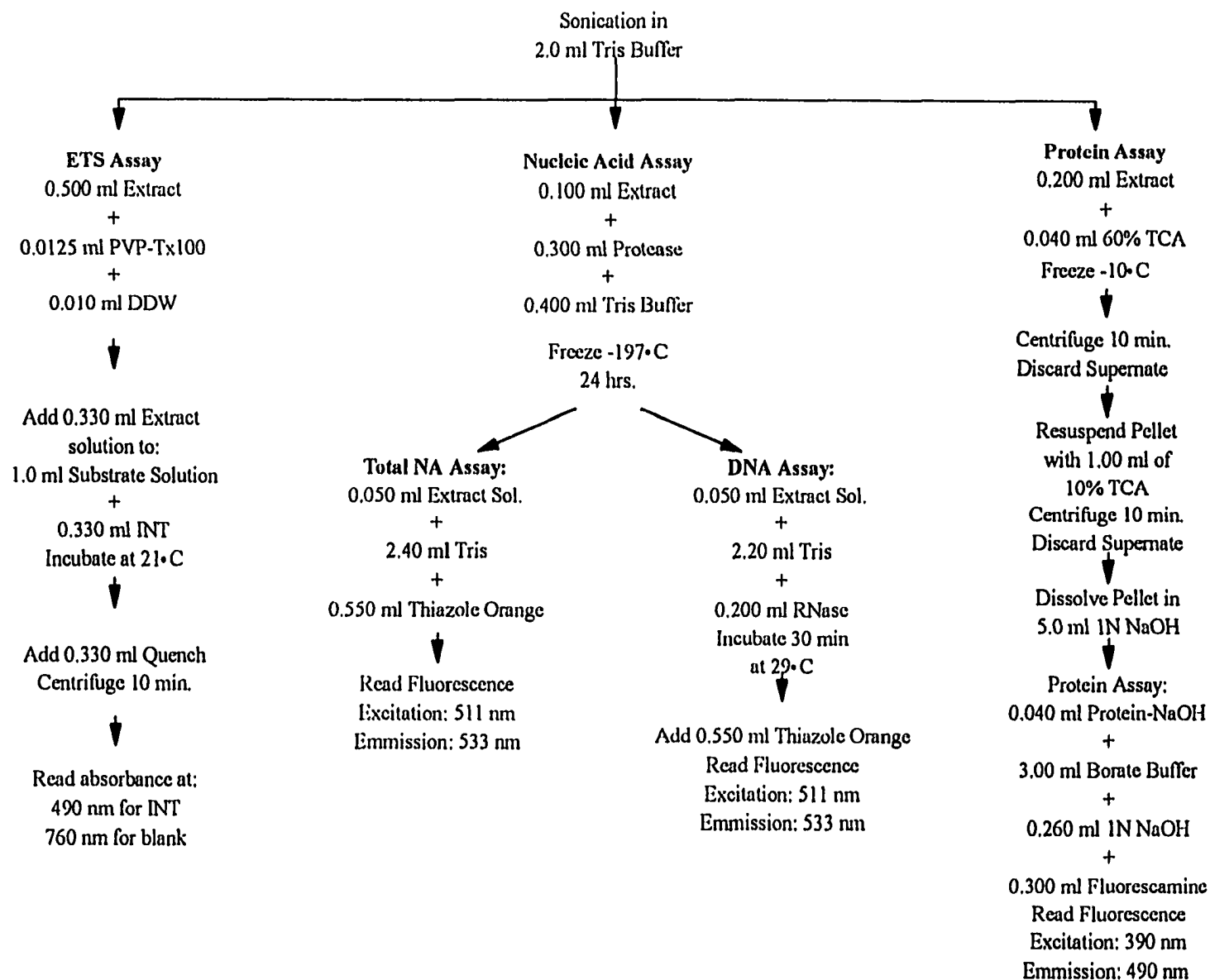
### **Extraction Technique**

Whole, individual larvae were homogenized in a small diameter (7.5 mm) glass culture tube containing 2.0 ml of Tris buffer (0.1322 CaCl<sub>2</sub>·7 H<sub>2</sub>O, 12.11 g Tris(hydroxymethyl)aminomethane-Sigma 7-9®, 5.84 g NaCl, pH 7.6) using a Fisher Model 50 Sonic Dismembrator operated at full power. Sonication was done on ice, in 7, 5 s bursts, separated by a 30 s cooldown.

### **Nucleic Acid Assay**

The procedure for the nucleic acid content assay is fully detailed in the previous chapter. After sonication, 0.100 ml of the extract was combined with 0.300 ml of proteinase-K (1 mg/ml) and 0.400 ml of Tris. The mixture was agitated then stored in liquid nitrogen overnight. The following day, samples were removed from the nitrogen and excess nitrogen was allowed to boil off for approximately 30 s. The vials were then placed into a 29° C water bath and allowed to incubate for 30 min. The samples were centrifuged for 10 min to remove cellular debris. The total nucleic acid concentration was measured by adding 0.050 ml of the extract solution to each of three replicate assay tubes containing 2.40 ml Tris. Immediately prior to the fluorescence

**Figure 3.1: Schematic representation of the three biochemical assays performed on individual gulf menhaden (*Brevoortia patronus*) larvae.**



reading, 0.550 ml of thiazole orange (TO) (21  $\mu$ M) was introduced while vortexing. DNA fluorescence was determined by the addition of 0.050 ml of the extract solution to each of three replicate assay tubes containing 2.20 ml Tris and 0.200 ml of Rnase solution (0.3 mg/ml Tris). The mixture was allowed to incubate for 30 min at 29° C. After the incubation was complete the assay tubes were placed in a room-temperature water bath for 2 min. Again, 0.550 ml of thiazole orange was added while vortexing, then the fluorescence was determined. All measurements of fluorescence were taken on a Photon Technology International Quantum Master-1 with an excitation wavelength of 511 nm and emission wavelength of 533 nm. Standard curves were run daily prior to the samples analysis using a range of 0.0 to ~0.50  $\mu$ g of DNA/assay and 0.0 to ~2.0  $\mu$ g RNA/assay. DNA standard was from calf thymus (Sigma, St. Louis, MO) and RNA standard from bakers yeast (Sigma, St. Louis, MO). Each replicate larval sample reading consisted of the average of 10 fluorescence readings taken once per second. For the calculation of nucleic acid content, the mean fluorescence of the three replicates was used in the equations. The calculations for nucleic acid content were:

$$DNA (\mu\text{g/larvae}) = \frac{DNA_{FL} - DNA_{Intercept}}{DNA_{slope}} \times \frac{Cryo. Vol}{DNA Ext.} \times \frac{Son. Vol}{NA Ext.}$$

$$RNA (\mu\text{g/larvae}) = \frac{Total_{FL} - DNA_{FL} - RNA_{Intercept}}{RNA_{Slope}} \times \frac{Cryo. Vol.}{Total Ext.} \times \frac{Son. Vol.}{NA Ext.}$$

Where  $DNA_{FL}$  and  $Total_{FL}$  are the fluorescence readings for the DNA and total nucleic acid assay,  $DNA_{Slope}$  and  $DNA_{Intercept}$  are the slope and intercept of the DNA standard

curve. Those for the RNA standard curve are similarly subscripted. “Cryo. Vol.” is the combined volume of the frozen protease, larval extract and Tris. “DNA” and “Total extract” is the amount of the “cryo vol.” used in the assay. “Son. Vol.” is the sonication volume and “NA Ext.” is the amount of sonication volume set aside for the nucleic acid assay.

### **Electron Transport System Assay**

The ETS assay is modified from Packard and Williams (1981). The Packard and Williams assay is based on the production of formazan (reduced tetrazolium) by ETS enzymes when provided with succinate, NADH and NADPH as electron donors (Packard, 1985). The quantity of formazan production is determined spectrophotometrically. Three modifications used in this study were necessary: (1) to make the extraction procedure compatible with the nucleic acid assay (change buffer from phosphate to Tris), (2) to remove steps and reagents necessary for the measurement of phytoplankton ETS activity but not that of fish larvae (pigment blanks and the addition of sodium cyanide to inhibit photosynthetic electron transport), and (3) to increase the sensitivity by scaling down the assay and reducing the blanks. A summary of the new assay and its calibration against measured respiration rates is given in Dortch et al. (1996) and a more complete description will be presented in Dortch (in prep).

The following is the protocol followed in this research. Substrate buffer, 9.0 g disodium succinate hexahydrate  $C_4H_4O_4Na_2 \cdot 6H_2O$  (Sigma, St. Louis, MO) dissolved in 250 ml Tris buffer, was stored frozen in appropriate aliquots. Iodonitrotetrazolium

Violet (INT) solutions were created by adding 1.0 g of INT ( $C_{19}H_{13}ClIN_5O_2$ , MW 505.7, U.S Biochemical, Cleveland, OH) to 500 ml distilled deionized water (DDW) and stirring for 1 h. The solution was then filtered through a pre-combusted glass fiber filter to remove the un-dissolved particulates. Stock solutions of INT were stored frozen in appropriate aliquots. A quench solution used to stop the reaction consisted of 17.25 ml  $H_3PO_4$ , 232.75 ml DDW and 250 ml 37% formaldehyde and was stored at room temperature. Triton X-100- Polyvinylpyrrolidone solution (TX-100-PVP) was 2.0 ml TX-100, 1.5 g polyvinylpyrrolidone (ave. mol wt. 10,000) and 18.5 mg  $MgSO_4 \cdot 7H_2O$  brought to 25 ml with Tris buffer. The TX-100-PVP solution was stored frozen. On the day of the assay, the reagents were thawed and stored on ice, NADH ( $\beta$ -nicotinamide adenine dinucleotide, reduced form,  $C_{21}H_{27}N_7O_{14}P_2Na_2$ , Sigma, St. Louis, MO) and NADPH ( $\beta$ -nicotinamide adenine dinucleotide phosphate, reduced form,  $C_{21}H_{27}N_7O_{17}P_3Na$ , Sigma, St. Louis, MO) were added to the substrate buffer at 625  $\mu g/ml$  for NADH and 208  $\mu g/ml$  for NADPH.

For the assay, 0.500 ml of the sonication extract was transferred to a vial containing 0.0125 ml of TX-100-PVP and 0.010 ml DDW and the vial was briefly agitated. The mixture was then stored on ice. After a sufficient number of samples had been sonicated (usually 10 larvae per run of the assay), 0.330 ml of the extract, TX-100-PVP, distilled water mixture was added to an assay tube containing 1.0 ml of the substrate solution and 0.330 ml of INT solution, then incubated at 21° C for color development. To ensure that each extract sample was incubated for an identical amount of time, each series of assays and reagent blanks was completed using the following

steps. A timer was started when 0.330 ml of the first sample extract was added to an assay tube. The assay tube was briefly agitated. After 30 s, 0.330 ml of the second samples extract was added to the second assay test tube and the tube briefly agitated. This process was repeated at 30 s intervals until all samples and reagent blanks had been added to the assay tubes. After adequate color development occurred in the first assay tube (usually 6 min), the total time elapsed was noted and 0.330 ml of the quench solution was added to the first assay tube and the tube was briefly agitated. At 30 s intervals, 0.330 ml of quench solution was added to the remaining assay tubes. After all assays had been quenched, the samples were centrifuged for 10 min and the absorbance read at 490 nm for INT and 760 nm for turbidity on a Varian DMA 80 UV/visible spectrophotometer. Two reagent blanks were run with each set of samples to control for the non-biological conversion of INT. Reagent blanks were identical to the samples except that the 0.500 ml of extract which was combined with TX-100-PVP was replaced with Tris buffer. Additional blanks, accounting for absorbance of the extract in the absence of INT, were not run. Earlier work has shown that this blank is small in fish larvae and it is proportional to the sample absorbance, therefore the sample absorbance was corrected by multiplying by 0.95 (Dortch, pers. comm.).

The calculation for the determination of ETS activity is:

$$\mu\text{l } O_2/\text{min}/\text{larvae} = \left( \frac{\left( \frac{COD - RB}{1.42} \right) \times S \times \left( \frac{H}{F} \right)}{T} \right) \times \left( \frac{Son.Vol.}{H} \right)$$

where  $COD = (ABS_{490 \text{ Sample}} - ABS_{760 \text{ Sample}}) * 0.95$ ,  $RB = (ABS_{490 \text{ Reagent Blk}} - ABS_{760 \text{ Reagent Blk}})$ ,  $S$ =assay volume,  $H$ =total homogenate saved for ETS assay (including TX-100-PVP and DDW),  $F$ =homogenate volume used in the assay,  $T$ =incubation time in minutes,  $Son. Vol.$ = sonication volume,  $1.42 = \text{ABS}/\mu\text{l O}_2/\text{ml}$  derived from the molar extinction of INT (Packard and Williams 1981).

### **Arrhenius Activation Energy**

Since marine poikilotherms are subjected to a wide range in temperature, mechanisms must exist to regulate enzymatic activity at the temperature extremes. One such mechanism is the alteration of biochemical structure of the enzyme. If such a mechanism is used by menhaden larvae, then larvae captured during cold periods would have structurally, as well as kinetically different enzymes than larvae captured during warm periods and the direct comparison of these would be inappropriate. To determine if structural modification is occurring in menhaden, the Arrhenius activation energy of three groups of laboratory reared menhaden larvae were compared (Packard et al., 1975). Menhaden eggs were collected from the field and the larvae reared to 15 days at 20° C. At day 15, the larvae were separated into three groups, group 1 was immediately transferred to liquid nitrogen, group 2 was transferred to an 18° C tank, and group 3 was transferred to a 22° C tank. Groups 2 and 3 were allowed to acclimate to the new temperature for 2 days, after which they were transferred to liquid nitrogen for ETS analysis. A total of 4 samples of larvae were obtained for each temperature with each sample consisting of 2 larvae for group 2 and 3 and 3 larvae for group 1. ETS activity of all groups was measured at incubation temperatures of 10°, 13°, 16°, 19°, 22° and 25° C.



Arrhenius activation energy,  $E_a$ , was calculated from the slope of the Arrhenius plots,  $\ln(\text{ETS activity})$  vs  $T^{-1}$  ( $^{\circ}\text{K} \times 1000$ ), according to Packard et al., (1975).

### **Protein Assay**

The protein assay was based on the procedure of Udenfriend et al. (1972). It is a fluorescent assay which measures the concentration of primary amines. For the protein assay, 0.200 ml of the sonication extract was placed into a conical tube with 0.040 ml of 60% trichloroacetic acid (TCA) to precipitate the proteins. The mixture was then frozen at  $-10^{\circ}\text{C}$  until sufficient numbers had accumulated. Prior to the measurement of protein concentration, the samples were thawed and centrifuged for 10 min. The supernate was discarded and the pellet resuspended with 1.00 ml of 10% TCA to remove any remaining Tris buffer. Tris is a quaternary amine that may react with the fluorescamine. The mixture was centrifuged for 10 minutes and the supernate discarded. The protein pellet was dissolved in 5 ml 1N NaOH and the protein-NaOH sample extract stored frozen. The day of the assay, the protein-NaOH sample extract was brought to room temperature, vortexed vigorously then centrifuged for 10 min. The protein assay was comprised of 3.0 ml of borate buffer (12.37g  $\text{H}_3\text{BO}_3$  / L DDW, pH adjusted to 7.0 with 4N NaOH), 0.260 ml 1 N NaOH, 0.040 ml protein-NaOH mixture and 0.300 ml fluorescamine (0.300 mg fluorescamine/ml acetone). The fluorescamine solution was added while vortexing immediately prior to the fluorescence reading. Three replicates were done for each protein-NaOH mixture. Fluorescence reading were taken on Photon Technology International Quantum Master-1 with excitation wavelength set at 390 nm and emission wavelength set at 475 nm (slit width 3 nm). Prior to sample analysis,

standard curves were run daily using a range of approximately 0.0 to 15.0 µg /assay of bovine serum albumin (Sigma, St. Louis, MO). Each replicate reading consisted of the average of 10 fluorescence readings taken once per second. For the calculation of protein content, the mean fluorescence of the three replicates was use in the calculations of protein content. The equation for protein content of the larvae is:

$$mg\ protein/larvae = \left( \left( \frac{Protein_{Fl} - Protein_{Intercept}}{Protein_{slope}} \right) \times \left( \frac{PV}{PV_{used}} \right) \times \left( \frac{Son.Vol}{Prot.Ext} \right) \right) \times 1000^{-1}$$

where Protein<sub>Fl</sub> is the fluorescence of the assay, Protein<sub>Intercept</sub> and Protein<sub>Slope</sub> are the intercept and slope of the protein standard curve, PV is the Protein-NaOH mixture volume, PV<sub>used</sub> is the amount of protein-NaOH mixture used in the assay, Son. Vol. is the sonication volume and Prot. Ext. is the amount of larval extract put aside for the protein assay.

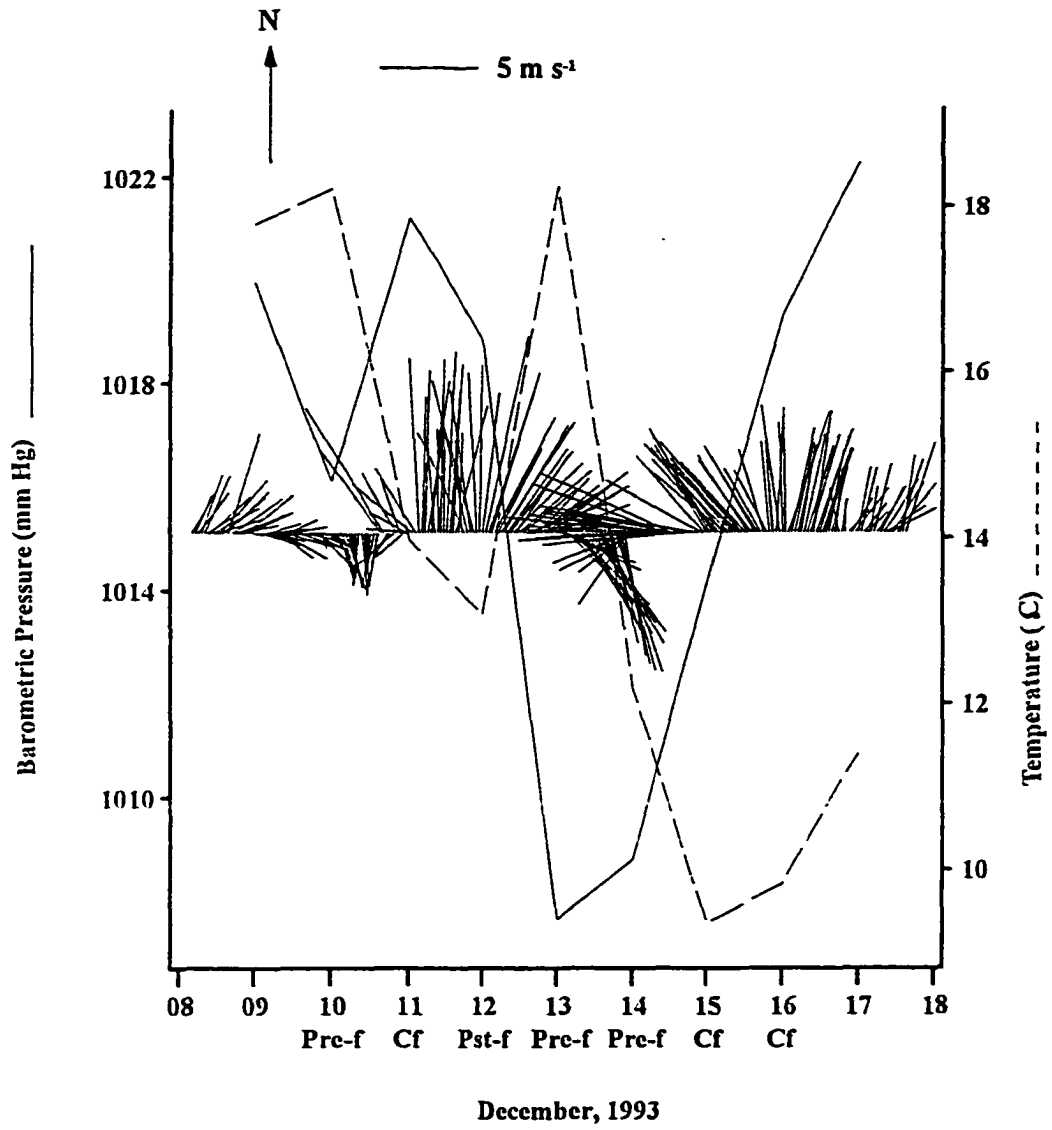
### Meteorological Data

The meteorological data used in this study came from a C-MAN weather buoy (GDIL) located near Grand Isle, LA, approximately 20 Km from the study site. The meteorological data consisted of hourly recordings of air and water surface temperature, wind speed and direction and barometric pressure. The southerly and westerly components of the wind direction were calculated. Positive values of a southerly wind component are indicative of a predominately southern wind, negative values indicate northerly winds. All wind data is presented using meteorological conventions. For analyses, daily means were calculated for air and surface temperature, wind direction,

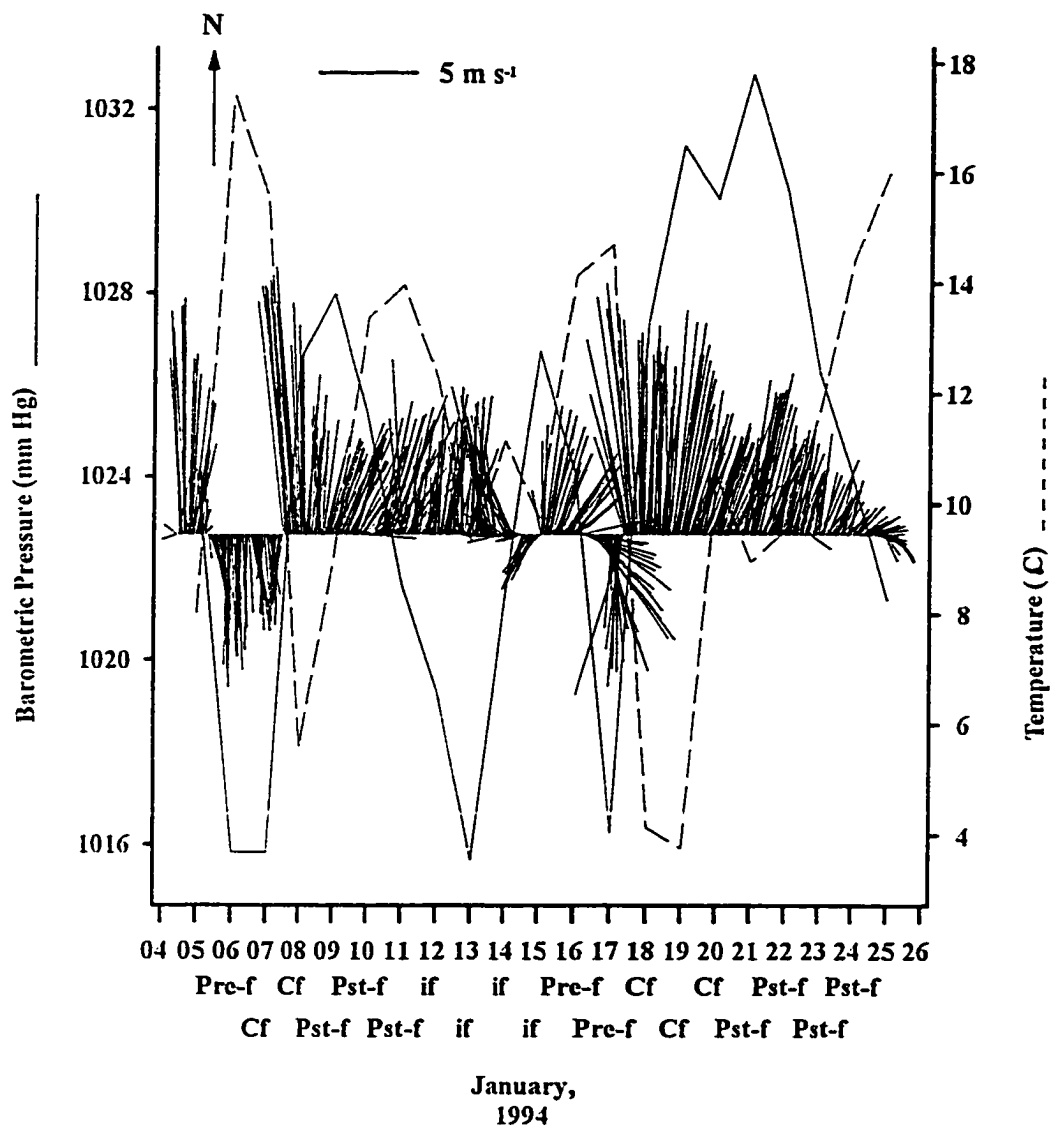
wind components and wind speed. Based on the wind speed and direction, air temperature and barometric pressure, each day of the six monthly sampling trips was classified in relation to cold front passage. Periods of predominately southern winds were classified as “pre-frontal”, a shift to northerly winds accompanied by a rise in barometric pressure and a drop in air temperature began a “cold-front” classification. The lessening of the intensity of the northerly component of the winds and a moderation in air temperature was classified as “post-frontal”. Light-intensity winds of varied direction were classified as “inter-frontal”. Under this classification scheme there were 13 pre-fronts (24 days), 12 cold-fronts (20 days), 10 post fronts (19 days), and 5 inter fronts (9 days) over the 72 total collection days. The wind vector, air temperature and barometric pressure plots with weather classification highlighted for each sampling trip are shown in Figure 3.2.

All statistical comparisons between years, among collection trips and among weather types were done using ANOVA and subsequent Tukey multiple comparisons with  $\alpha=0.05$ . For the examination of the inter-relationships of the various biochemical measures, if a biological reasons existed for the expectation of a linear or curvilinear relation, simple linear regression with appropriate transformations was used to examine relationships. If no particular relationship was expected, Pearsons product moment correlation was used.

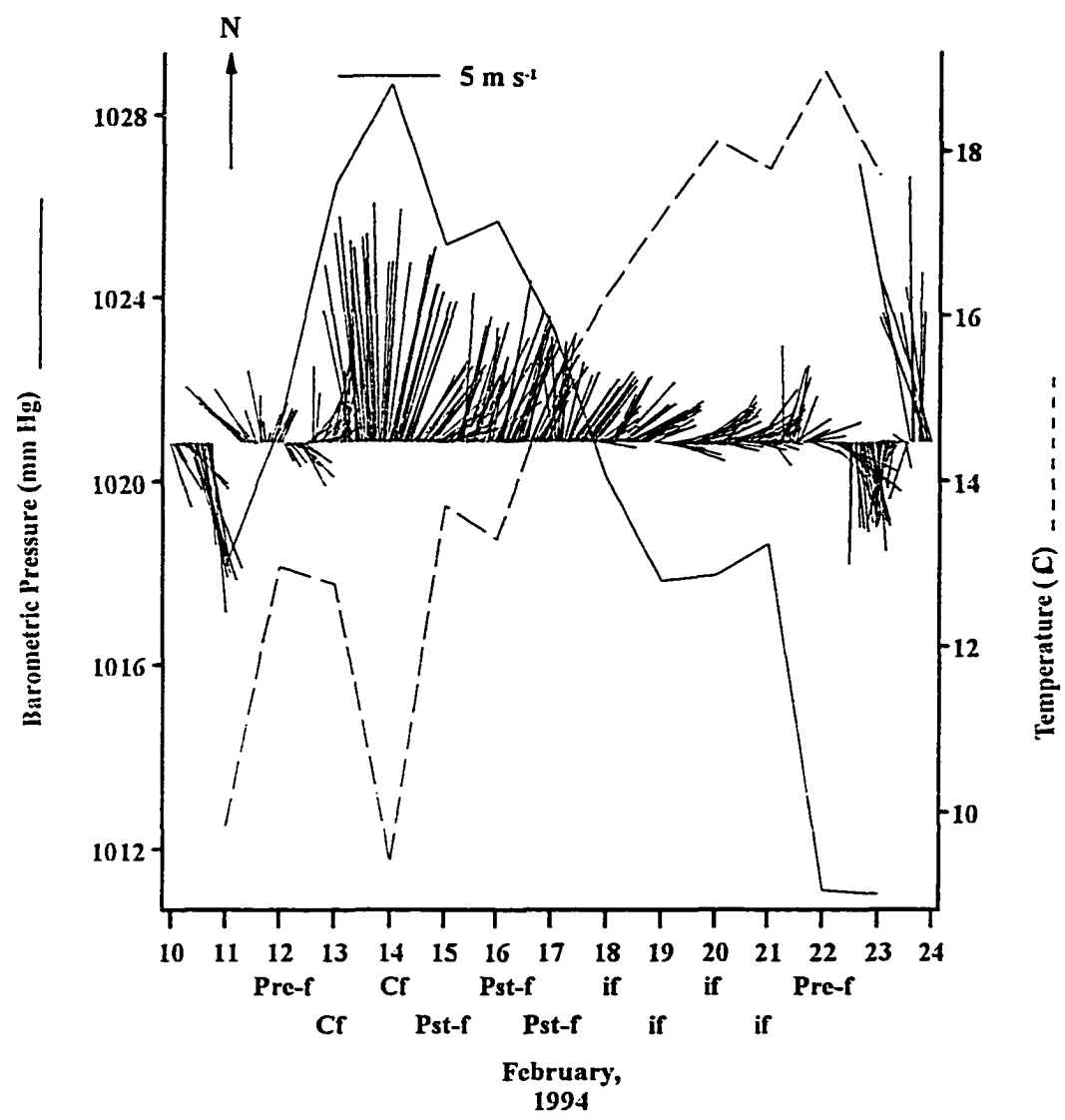
**Figure 3.2: Daily barometric pressure (mm Hg), temperature (°C) and wind characteristics for the 6 collection trips. Stick plots represent hourly wind speed and direction. Barometric pressure and temperature are daily means. Weather classification is denoted below each date of larval collection, Pre-f = Pre frontal, Cf = front, Pst-f = Post front and if = inter front.**



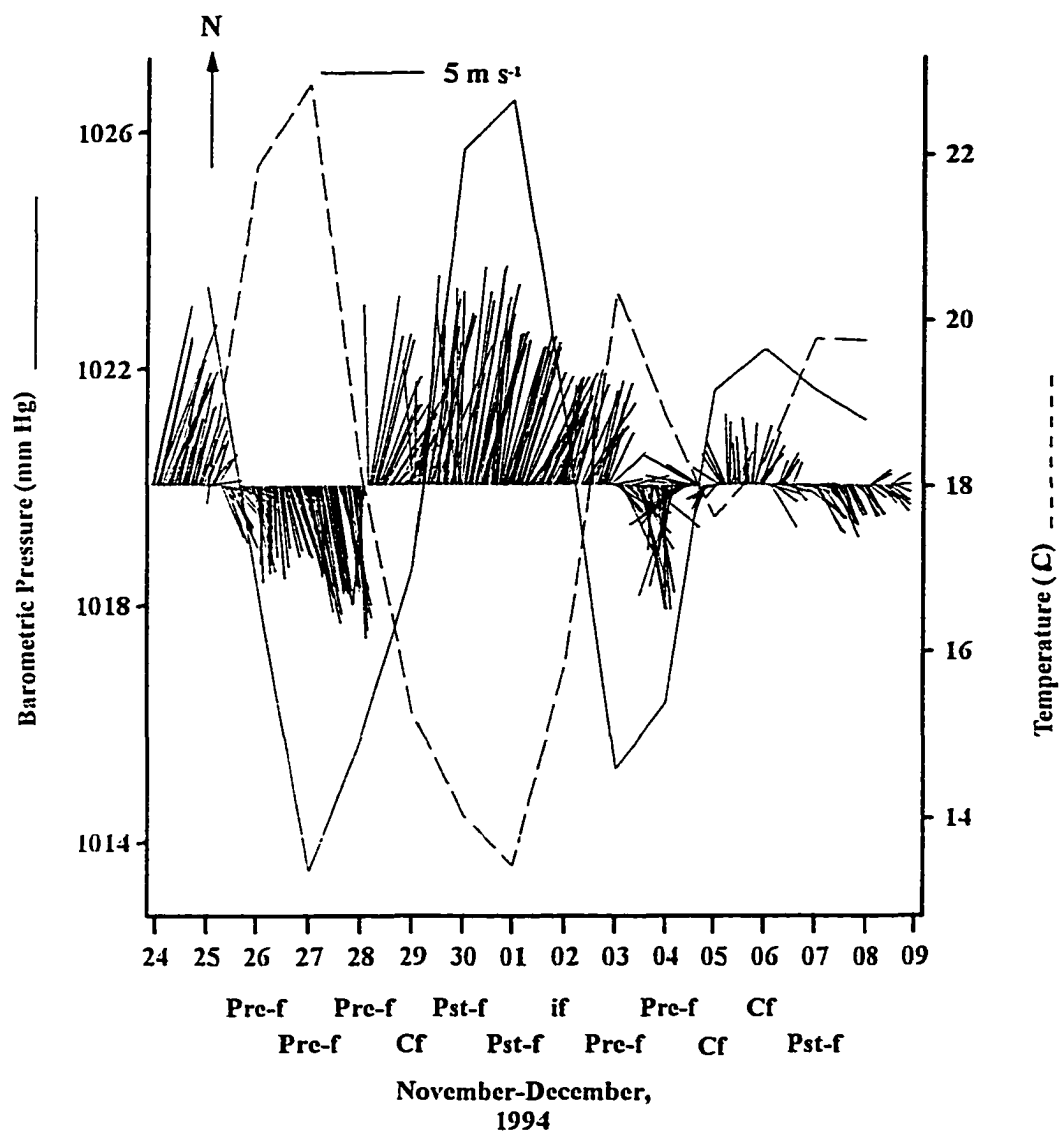
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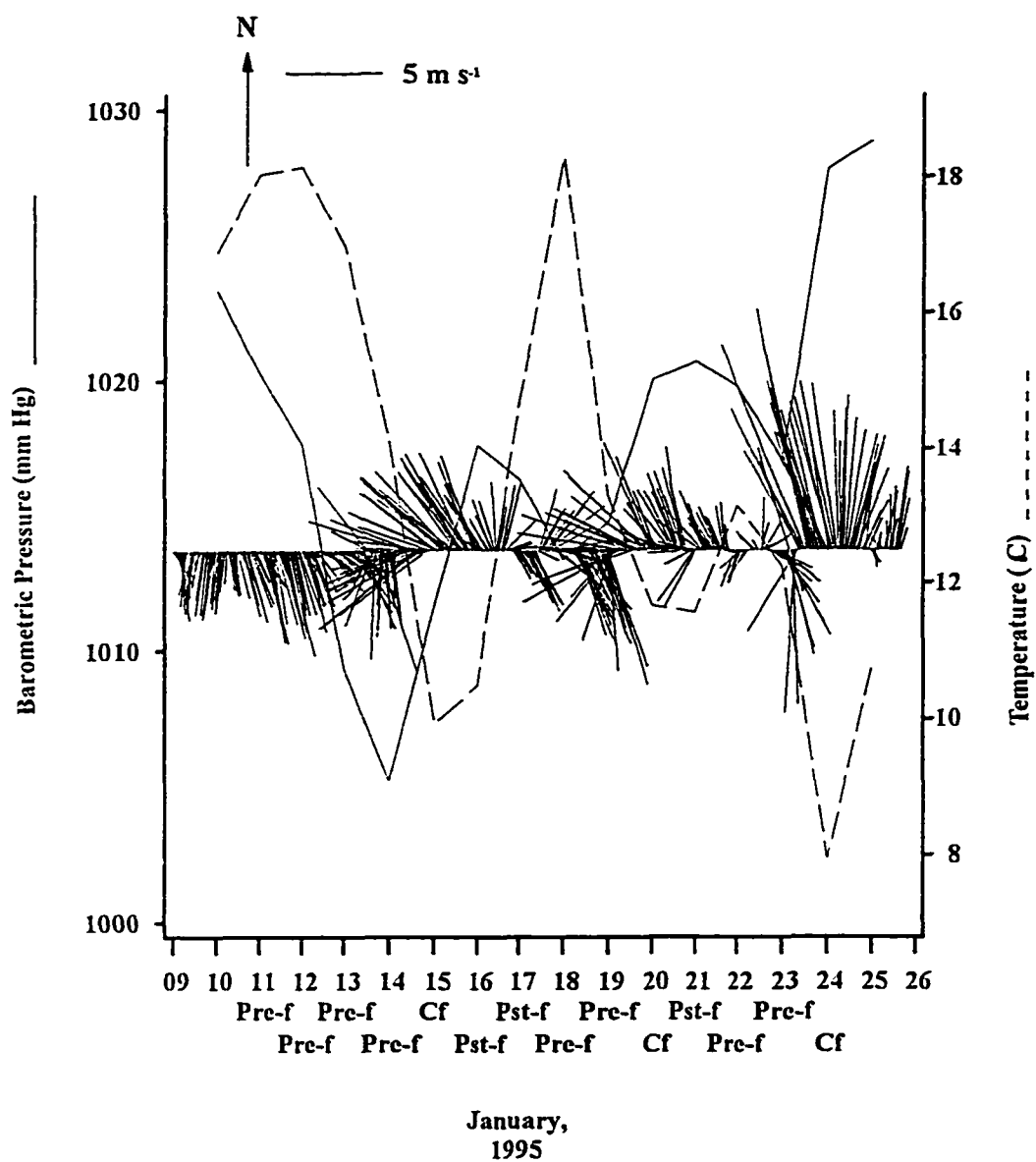


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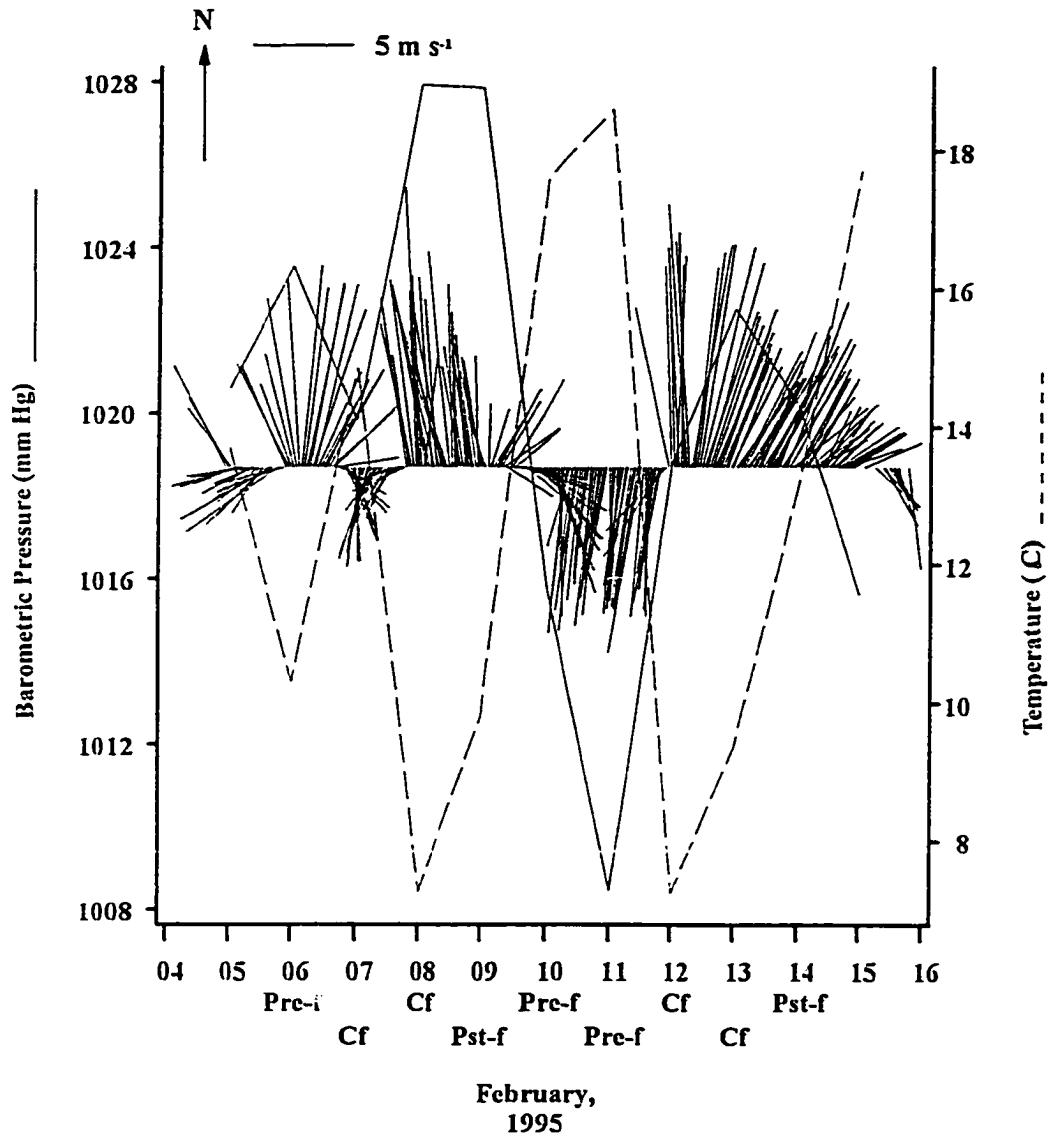


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(Fig. con'd.)



## RESULTS

A total of 1,349 larvae were collected and biochemically analyzed over the two field seasons, 716 larvae for the 1993-1994 season and 633 larvae for the 1994-1995 season. The complete data set is summarized in Table 3.2.

### Methodological Considerations

#### Effect of Chilling

The handling and chilling procedures used to slow larval movement prior to photography had no effect on the biochemical measures of condition. The three main measures of condition, protein, ETS activity and nucleic acid concentration were not affected by the chilling process. The protein content, nucleic acid content, RNA:DNA, ETS activity, and DNA-specific ETS activity of larvae chilled in an ice bath were not significantly different than larvae placed directly into liquid nitrogen (Table 3.3)

#### Arrhenius Activation Energies

The Arrhenius activation energies,  $E_a$ , calculated from the slopes of the Arrhenius plots (Figure 3.3) ranged from 17.1 to 20.5 (Table 3.4) and did not differ significantly between rearing temperature (ANCOVA,  $p=0.56$ ). Since the activation energy is independent of *in situ* temperature, the ETS assay presented here represents a quantification of the ETS enzyme concentration of the larvae.

### Relationships Among The Condition Indices

Several of the individual biochemical assays were linearly related to each other; the strongest relationships involving a measure related to organism size (protein, DNA or length) as the independent variable (Figure 3.4-3.10). ETS activity had a positive

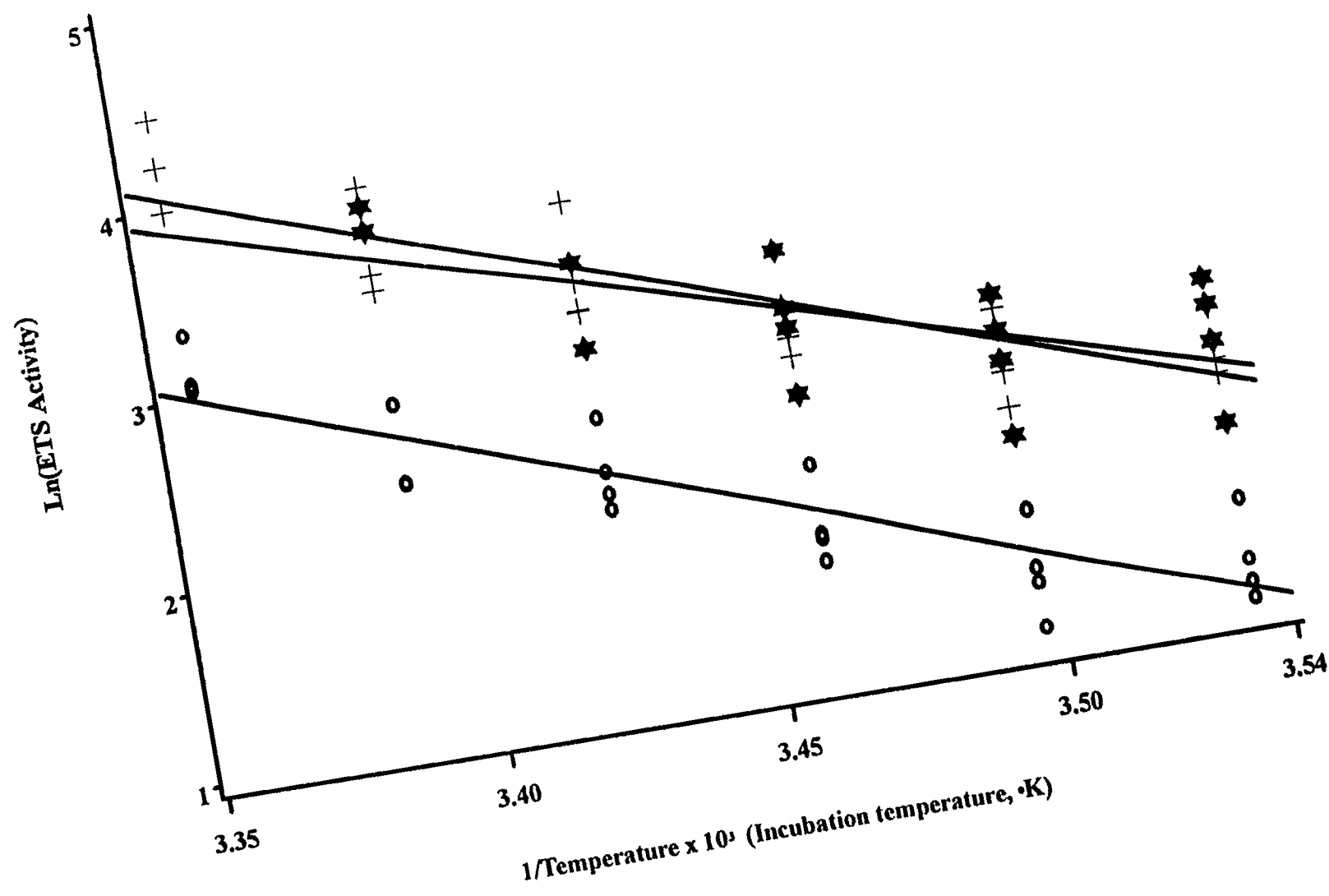
**Table 3.2 Summary statistics of the biochemical measures for all menhaden collected.**

<b>Biochemical Measure</b>	<b>n</b>	<b>Mean</b>	<b>Range</b>	<b>Standard Error</b>
RNA ( $\mu\text{g}/\text{larvae}$ )	1115	162.07	16.47-371.09	1.45
DNA ( $\mu\text{g}/\text{larvae}$ )	1115	43.48	10.07-128.63	0.352
RNA:DNA	1115	3.82	0.85-9.03	0.030
ETS $\mu\text{l O}_2/\text{min}/\text{larvae}$	1175	0.446	0.106-1.194	0.0034
Protein ( $\text{mg}/\text{larvae}$ )	1086	27.92	4.24-74.28	0.274

**Table 3.3: Results of the chilled vs non-chilled trial. Chilling the larvae prior to biochemical analysis had no effect on the biochemical measures of condition (ANOVA,  $\alpha=0.05$ )**

Biochemical Measure	Chilled (n=24)	Not Chilled (n=26)	P-value
RNA	157.48 $\pm$ 5.29	156.12 $\pm$ 5.14	0.85
DNA	43.46 $\pm$ 1.57	46.05 $\pm$ 1.96	0.30
ETS	0.411 $\pm$ 0.015	0.453 $\pm$ 0.017	0.08
Protein	10.90 $\pm$ 0.44	11.68 $\pm$ 0.45	0.32
RNA:DNA	3.67 $\pm$ 0.11	3.45 $\pm$ 0.11	0.18
ETS DNA <sup>-1</sup>	0.010 $\pm$ 0.0003	0.010 $\pm$ 0.0005	0.36

**Figure 3.3: Arrhenius plots showing the temperature dependence of the ETS activity of laboratory reared gulf menhaden larvae.  $\circ$ = 15 day old (DO) larvae reared at 20° C,  $\star$ = 17 DO larvae reared at 20° C with 2 day acclimation at 22° C,  $\dagger$ = 17 DO larvae reared at 20° C with 2 day acclimation at 18° C.**

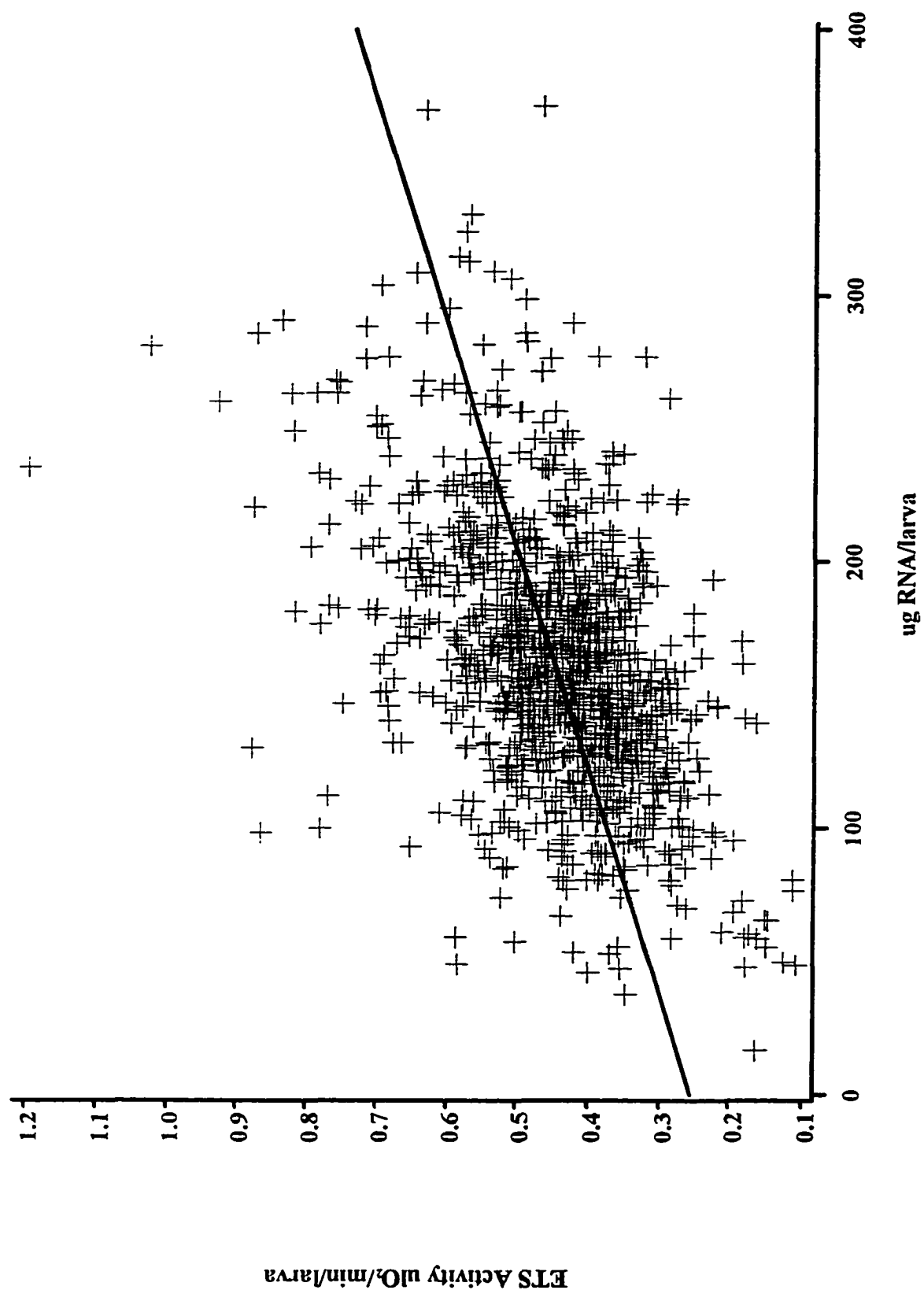


**Table 3.4: Results of the Arrhenius activation energy trials.  $E_a = -RS$ ,  $R=1.987$  kcal mol<sup>-1</sup> (gas constant) and  $S$ = slope of Arrhenius plot.  $E_a$  expressed in units of kcal mol<sup>-1</sup>.**

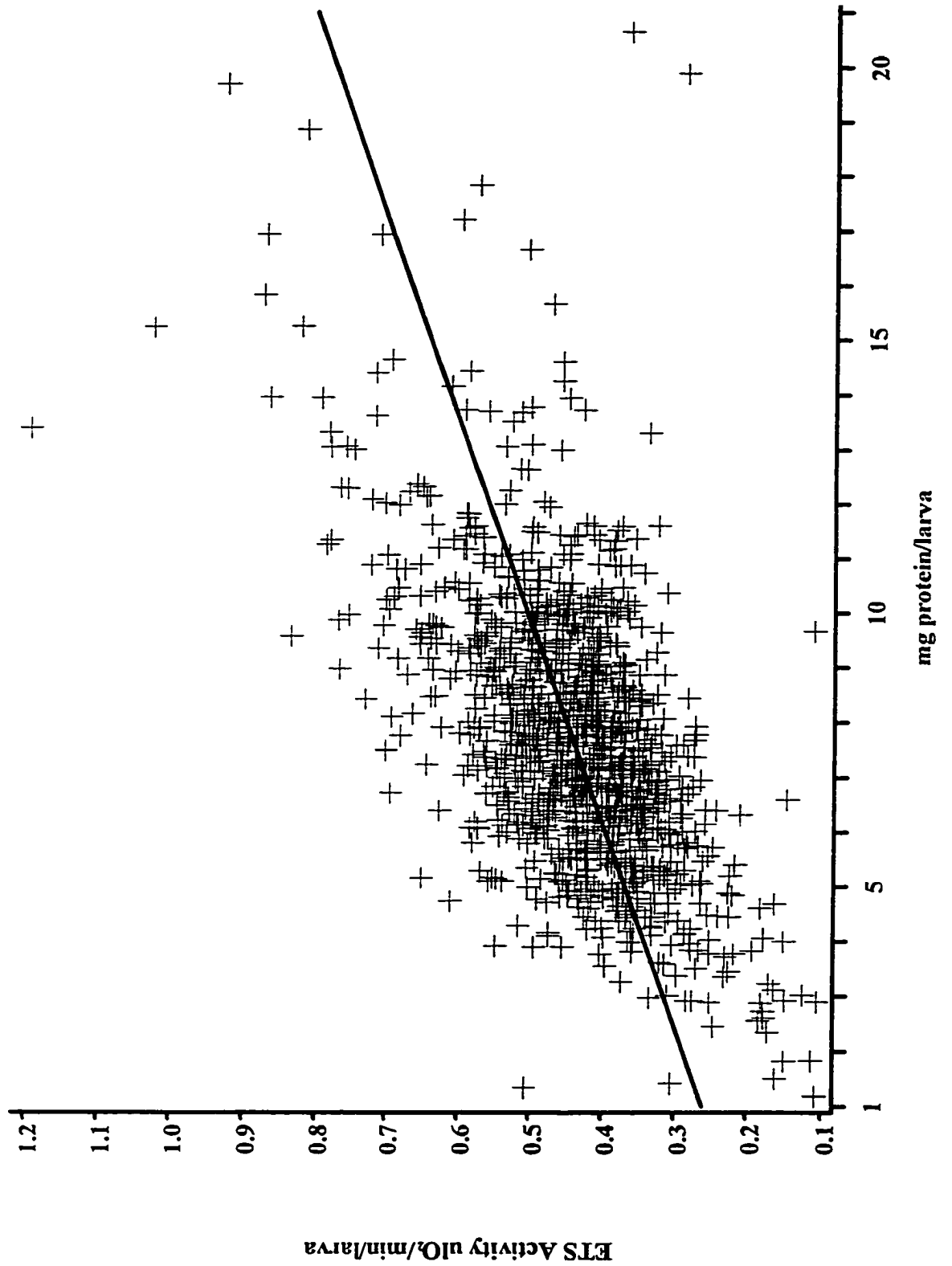
Age (Days after Hatch)	Rearing Temp °C	Slope Arrhenius Plot, S	Activation Energy $E_a = -RS$
15	22	-85.9774	17.083
17	20	-10.013	19.895
17	24	-10.299	20.464



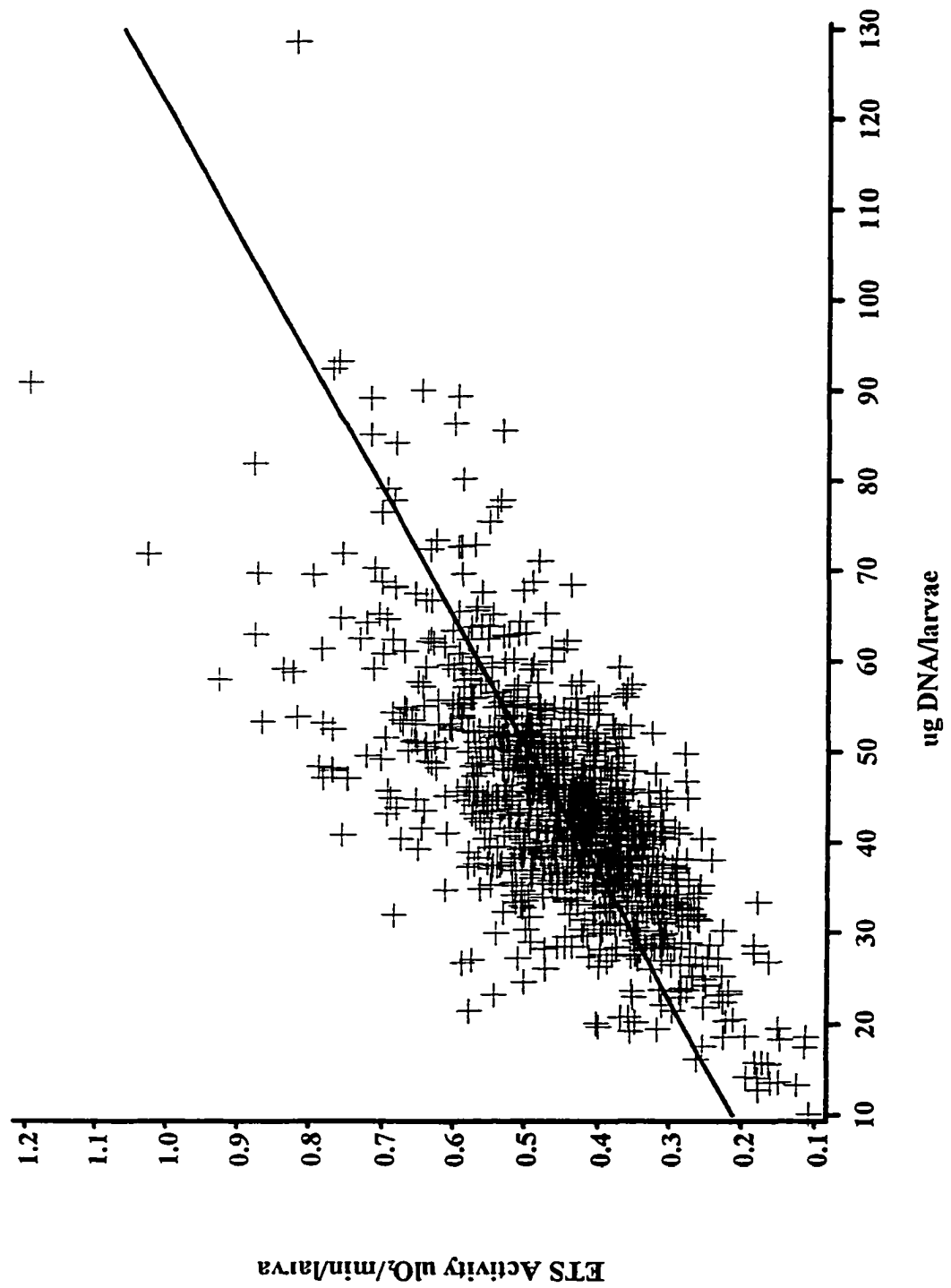
**Figure 3.4: Regression of ETS activity ( $\mu\text{l O}_2/\text{min}/\text{larva}$ ) on RNA ( $\mu\text{g}/\text{larva}$ ).  
ETS=0.26 + 0.0011(RNA),  $P<0.001$ ,  $r^2=0.22$ .**



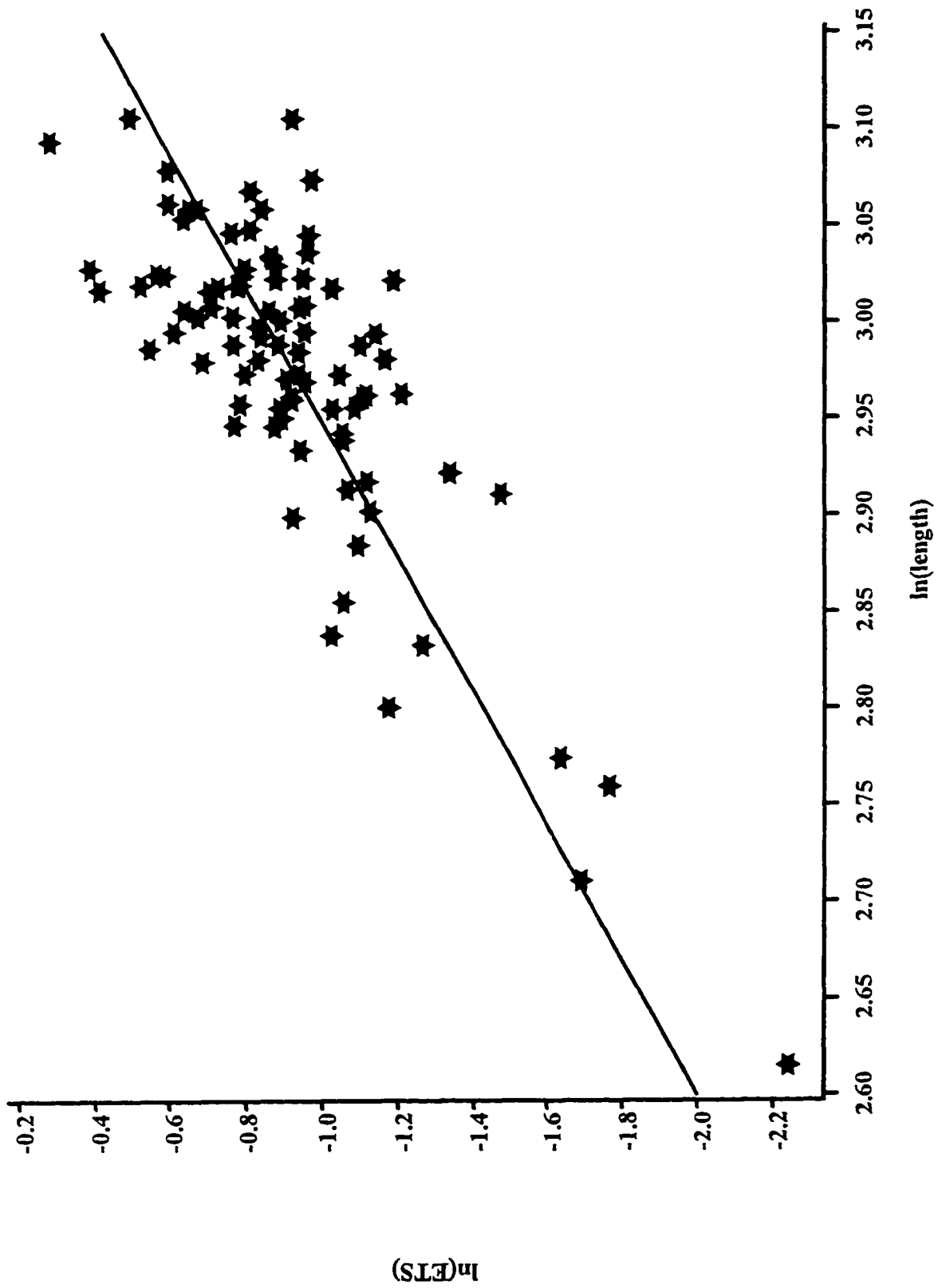
**Figure 3.5: Regression of ETS activity ( $\mu\text{l O}_2/\text{min}/\text{larva}$ ) on protein ( $\text{mg}/\text{larva}$ ).  
ETS =  $0.23 + 2.8 \times 10^{-6}(\text{Protein})$ ,  $P < 0.001$ ,  $r^2 = 0.33$ .**



**Figure 3.6: Regression of ETS activity ( $\mu\text{l O}_2/\text{min}/\text{larva}$ ) on DNA ( $\mu\text{g}/\text{larva}$ ).  
ETS=0.13 + 0.007(DNA),  $P<0.001$ ,  $r^2=0.46$ .**

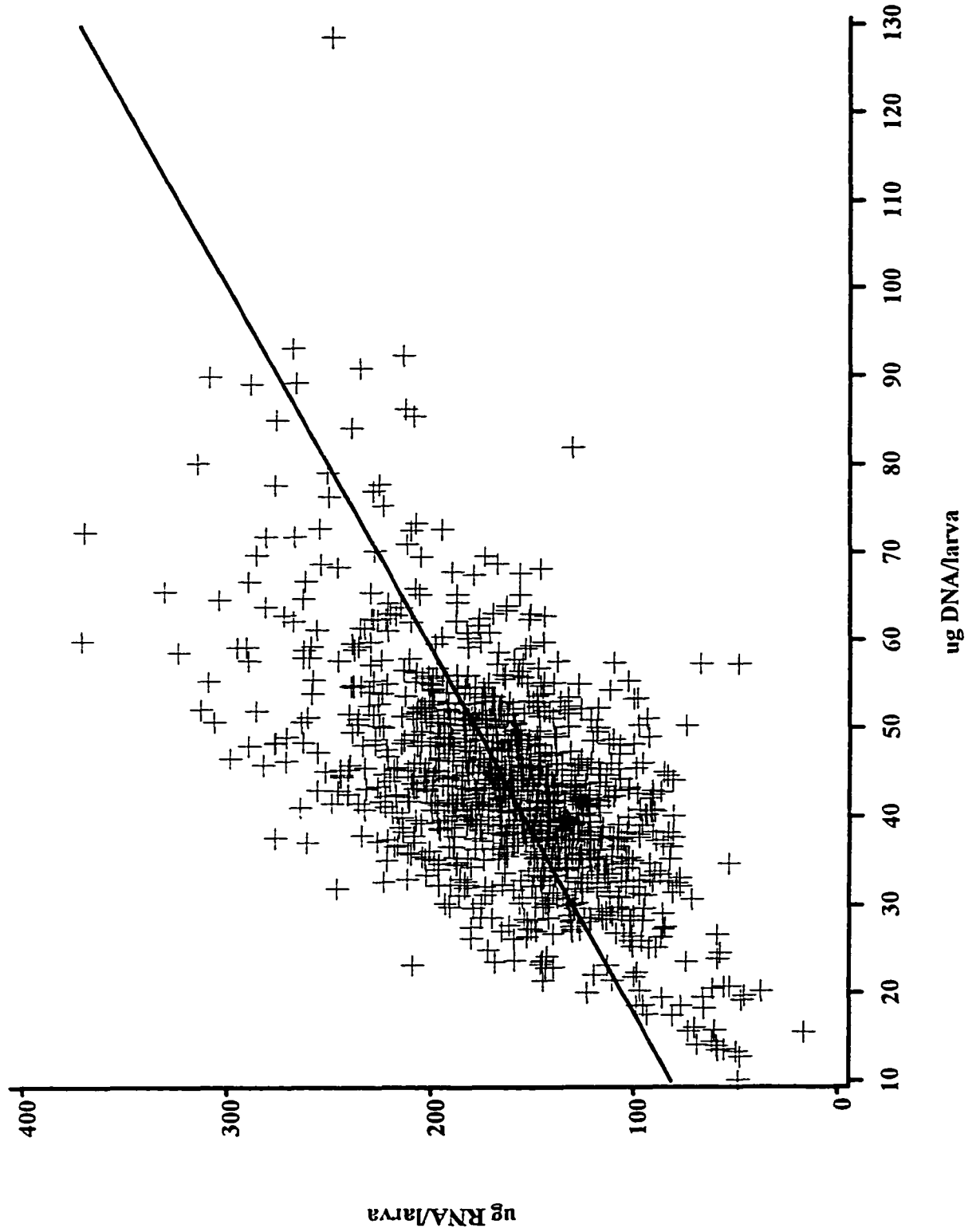


**Figure 3.7: Regression of ln(ETS activity) on ln(length).  $\ln(\text{ETS}) = -9.47 + 2.87\ln(\text{length})$ ,  $P < 0.001$ ,  $r^2 = 0.64$ .**

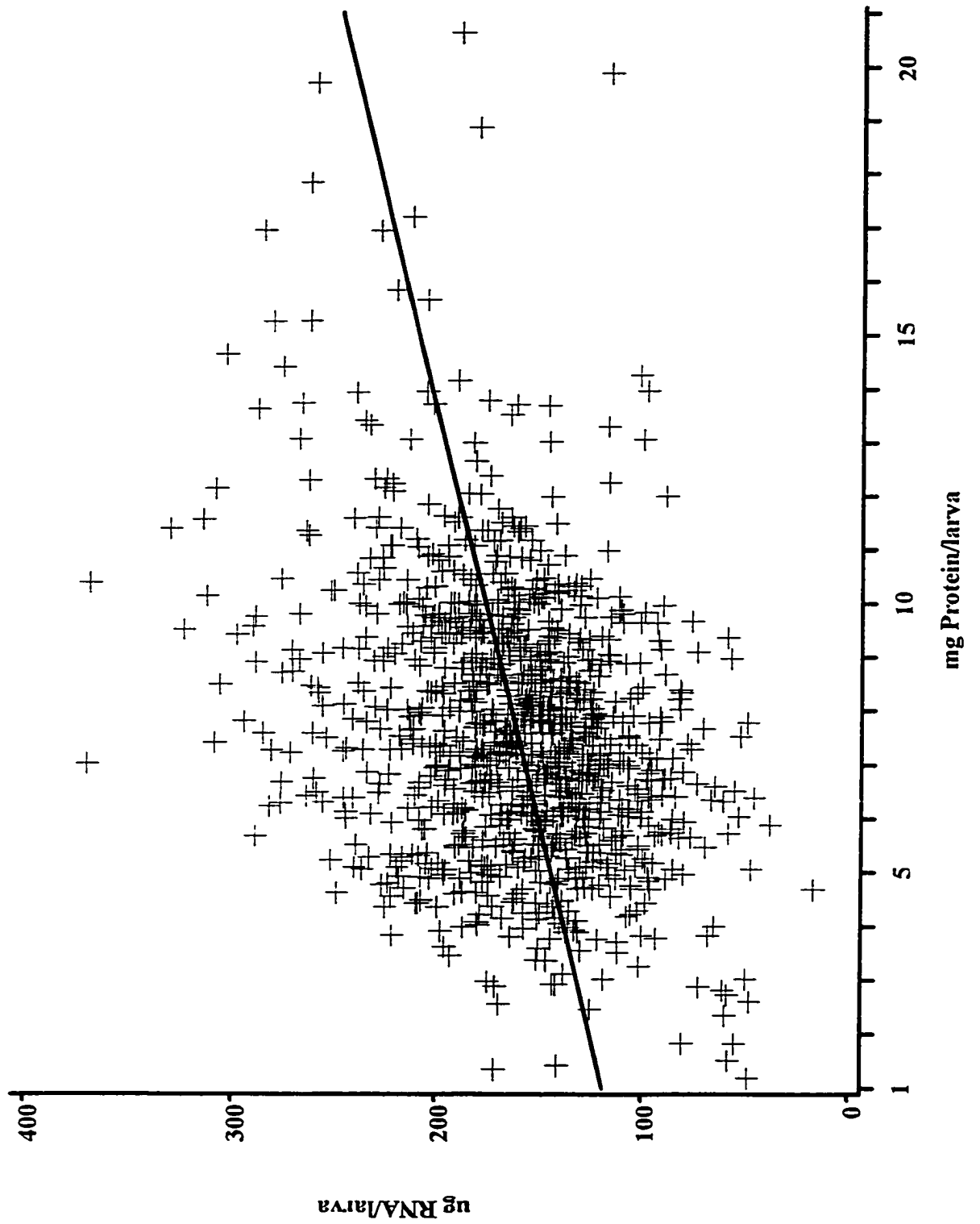




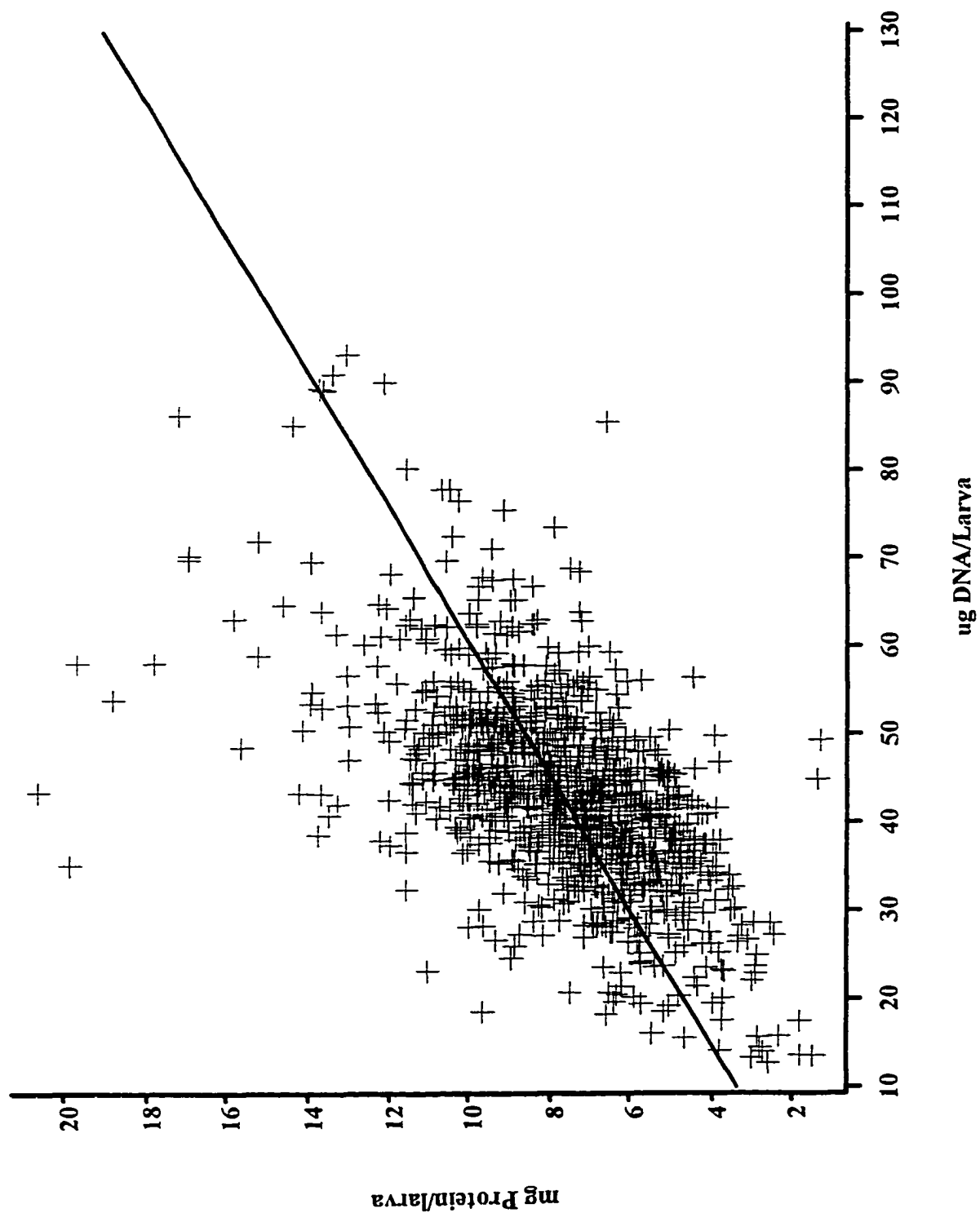
**Figure 3.8: Regression of RNA ( $\mu\text{g}/\text{larva}$ ) on DNA ( $\mu\text{g}/\text{larva}$ ).  $\text{RNA}=50.27 + 2.61(\text{DNA})$ ,  $P<0.001$ ,  $r^2=0.35$ .**



**Figure 3.9: Regression of RNA ( $\mu\text{g/larva}$ ) on Protein ( $\text{mg/larva}$ ).  $\text{RNA} = 113.6 + 0.006 (\text{protein})$ ,  $P < 0.001$ ,  $r^2 = 0.10$ .**



**Figure 3.10: Regression of protein (mg/larva) on DNA ( $\mu\text{g/larva}$ ). Protein=2079.8 +130.7(DNA),  $P<0.001$ ,  $r^2=0.34$ .**



linear relationship to RNA ( $p=0.001$ ,  $r^2=0.21$ ), protein ( $p=0.0001$ ,  $r^2=0.34$ ) and DNA ( $p=0.0001$ ,  $r^2=0.46$ ). ETS activity showed an exponential increase with larval length, but the relationship between the two variables is generally plotted as a logarithmic function (Savenkoff et al., 1996). There was a positive linear relationship between RNA and DNA ( $p=0.001$ ,  $r^2=0.35$ ), the regressions of RNA to protein and RNA to length, although significant, were weak ( $r^2=0.10$ ). Both protein and DNA were related to length ( $r^2=0.21$  for protein,  $r^2=0.36$  for DNA) as well to each other ( $p=0.0001$ ,  $r^2=0.34$ ). The relationships between length and protein as well as the relationship between length and DNA seem to be logarithmic but examination of the residuals showed no indication of lack of fit and the logarithmic model did not improve the amount of explanatory power of the model.

Protein specific ETS activity and  $\text{RNA protein}^{-1}$  were positively correlated ( $r=0.68$ ), however, the correlation of  $\text{RNA:DNA}$  to  $\text{ETS DNA}^{-1}$  was low ( $r=0.22$ ).  $\text{ETS DNA}^{-1}$  and  $\text{RNA protein}^{-1}$  were only weakly correlated ( $r=-0.12$ ) and the relation between  $\text{RNA:DNA}$  and  $\text{ETS protein}^{-1}$  was only slightly greater ( $r=0.36$ ).  $\text{RNA:DNA}$  values were strongly correlated to  $\text{RNA protein}^{-1}$  ( $r=0.61$ ). The size dependent aspect of the condition indices was evident in the negative correlation between  $\text{RNA:DNA}$  and length ( $r=-0.24$ ), a somewhat stronger correlation occurred between  $\text{RNA protein}^{-1}$  and length ( $r=-0.46$ ). There was no significant correlation between length and  $\text{ETS protein}^{-1}$  or  $\text{ETS DNA}^{-1}$ .

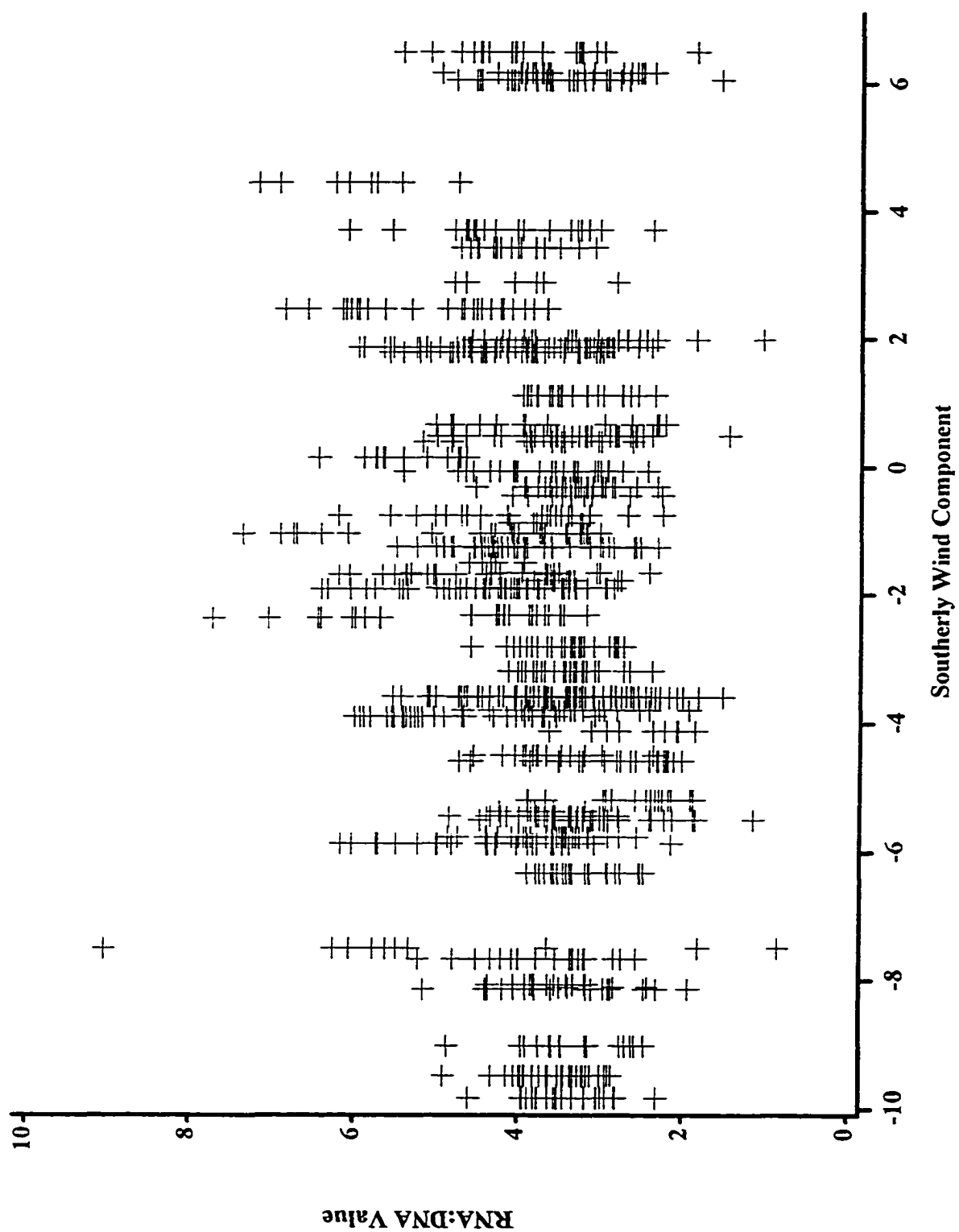
There was only weak correlation between all the biochemical measures of condition and the standard meteorological and environmental variables, mean wind speed

and direction and surface water and air temperature. RNA:DNA and RNA protein<sup>-1</sup> were positively correlated with the southerly wind component ( $r=0.13$  for RNA protein<sup>-1</sup> and  $r=0.16$  for RNA:DNA, Figure 3.11-3.12) and negatively correlated with the westerly wind component ( $r=-0.22$  RNA protein<sup>-1</sup>,  $r=-0.24$  RNA:DNA) but all correlations were weak.

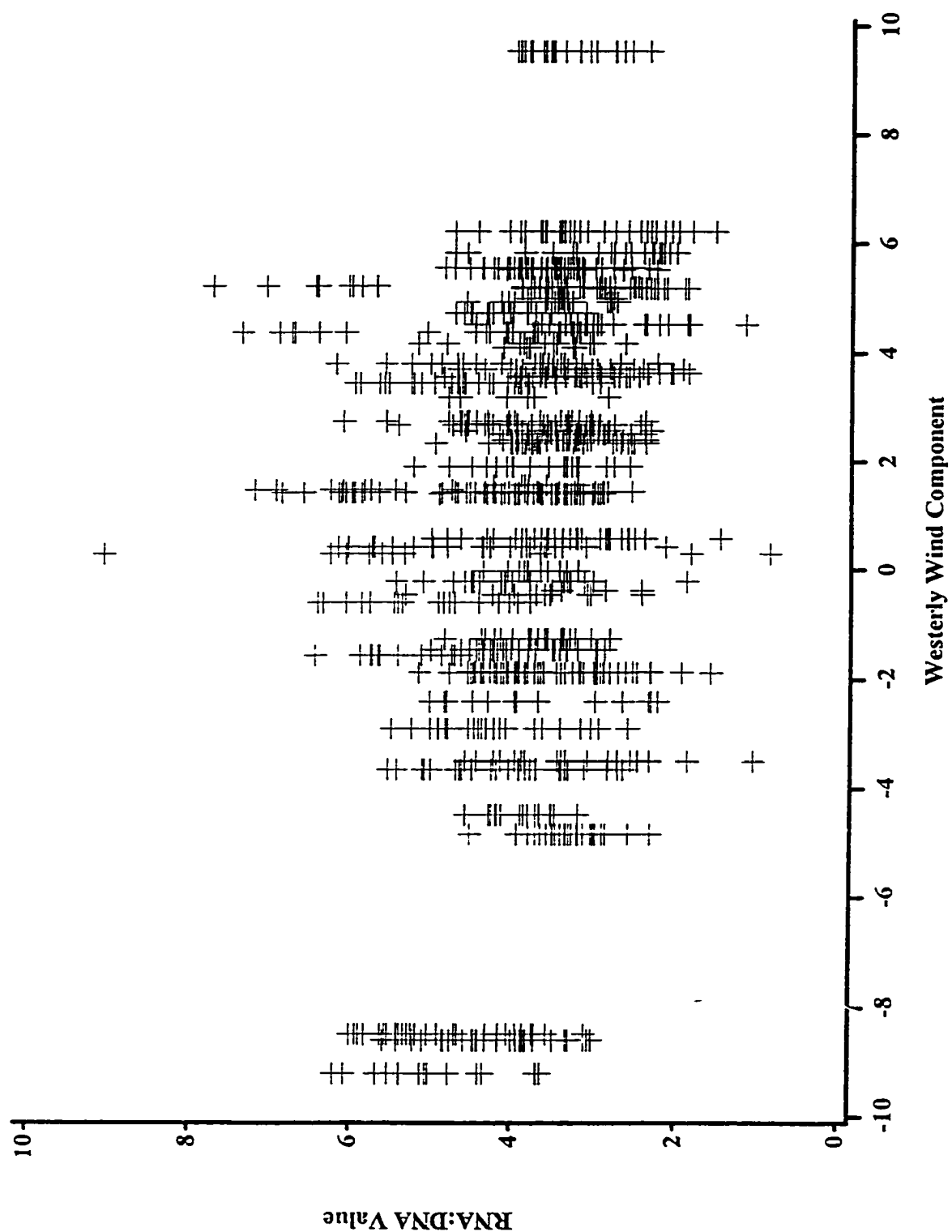
However, in terms of weather classification (pre-frontal, cold-front, post-front and inter-frontal) all of the biochemical indices of condition were greatest during the pre-frontal period (Figure 3.13-3.16). RNA:DNA values were significantly higher during the pre-frontal periods than during any other classification period. RNA protein<sup>-1</sup> values were significantly higher during pre-frontal period when compared to post-frontal and inter-frontal periods, it was also higher than cold-front periods, but not significantly. Protein specific ETS activity and DNA specific ETS activity were significantly higher during the pre-frontal than during the inter-frontal periods. Although both size corrected measures of ETS activity were higher than both cold-front periods and post-front periods, there was no significant difference. Total RNA per larvae was higher during pre-frontal than either post- or inter-frontal. Condition indices always were at their highest during pre-frontal, followed exclusively by levels during the cold-front periods. Inter-front levels and post-front levels were exclusively lowest and never differed significantly from each other. In terms of DNA, cold-front larvae were larger than inter-frontal but DNA content did not differ among pre-frontal, post-frontal and inter-frontal. Otherwise, weather classification had no relationship to the size of the larvae, based on



**Figure 3.11 Scatter plot of RNA:DNA values versus the southerly wind component. Negative values of the southerly component indicate a predominately northern wind, positive values indicate a predominately southern wind. The southerly component is scaled to wind speed.**

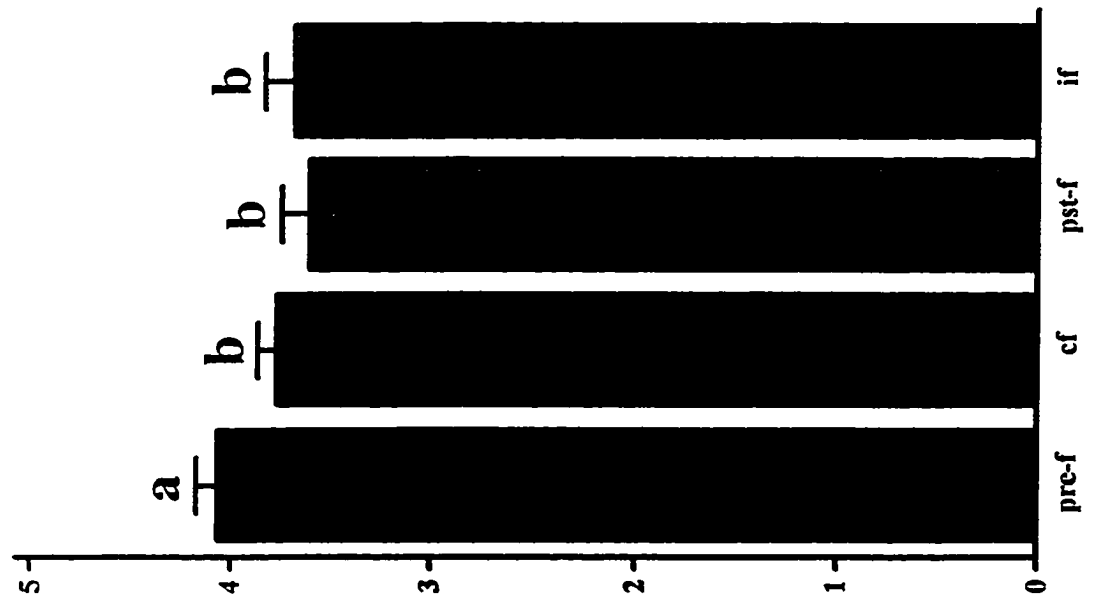


**Figure 3.12: Scatter plot of RNA:DNA values versus the westerly wind component. Negative values of the westerly component indicate a predominately eastern wind, positive values indicate a predominately western wind. The westerly component is scaled to wind speed.**

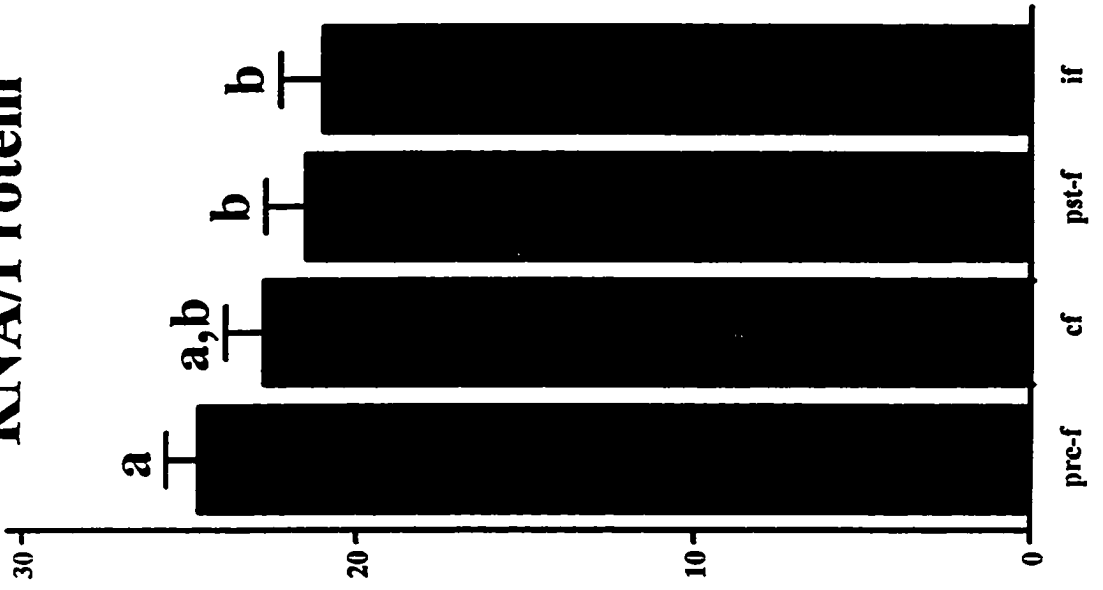


**Figure 3.13: Comparison of RNA:DNA and RNA Protein<sup>-1</sup> ( $\mu\text{g}/\text{mg}$ ) among weather classification variables. Pre-f=Pre-Frontal, CF=Cold-Front, Pst-F=Post-Front, If=Inter-Front. Error bars represent 1 standard error. Histogram bars denoted with the same letter are not significantly different (ANOVA, Tukey multiple comparison,  $\alpha=0.05$ ).**

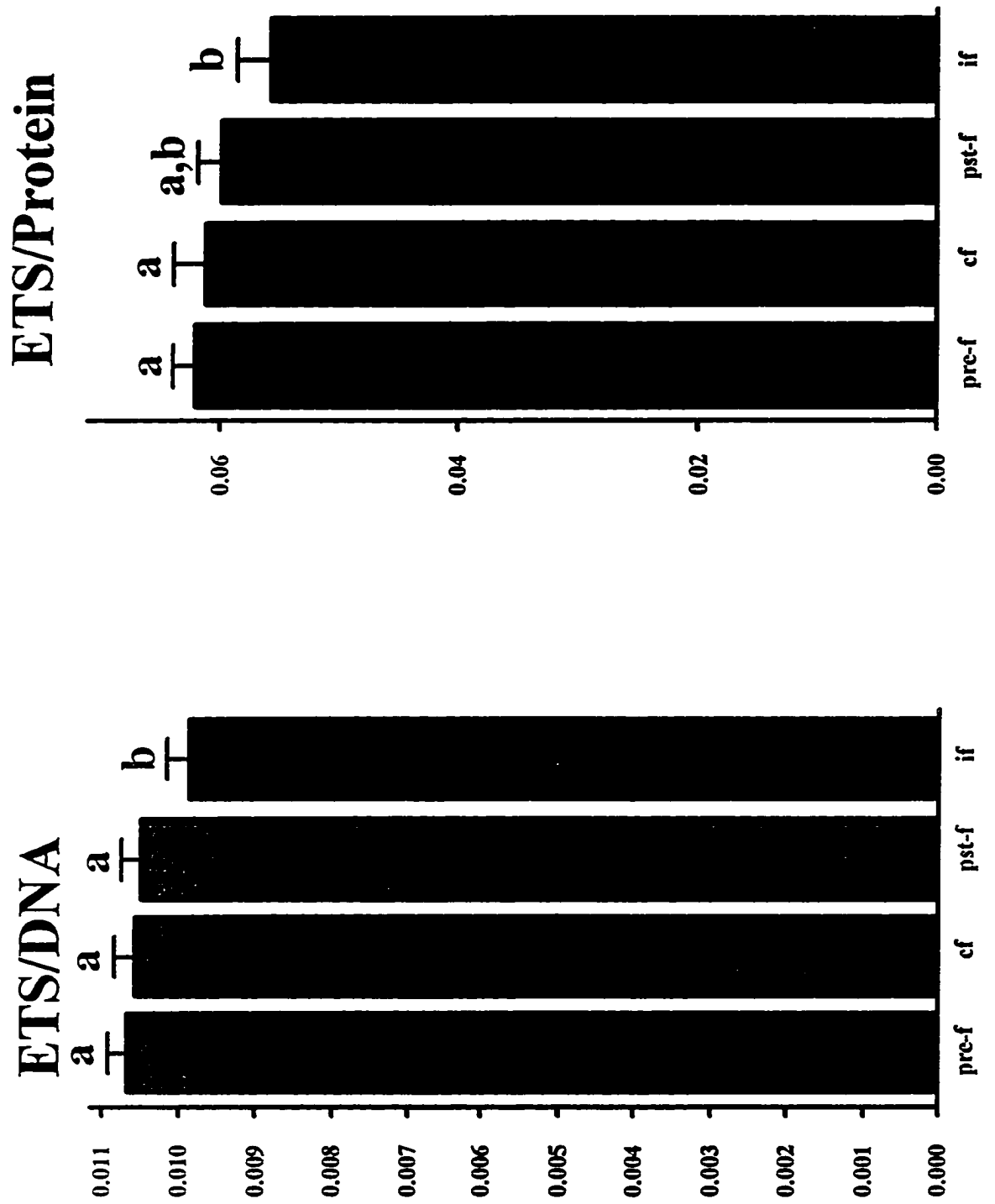
## RNA/DNA



## RNA/Protein

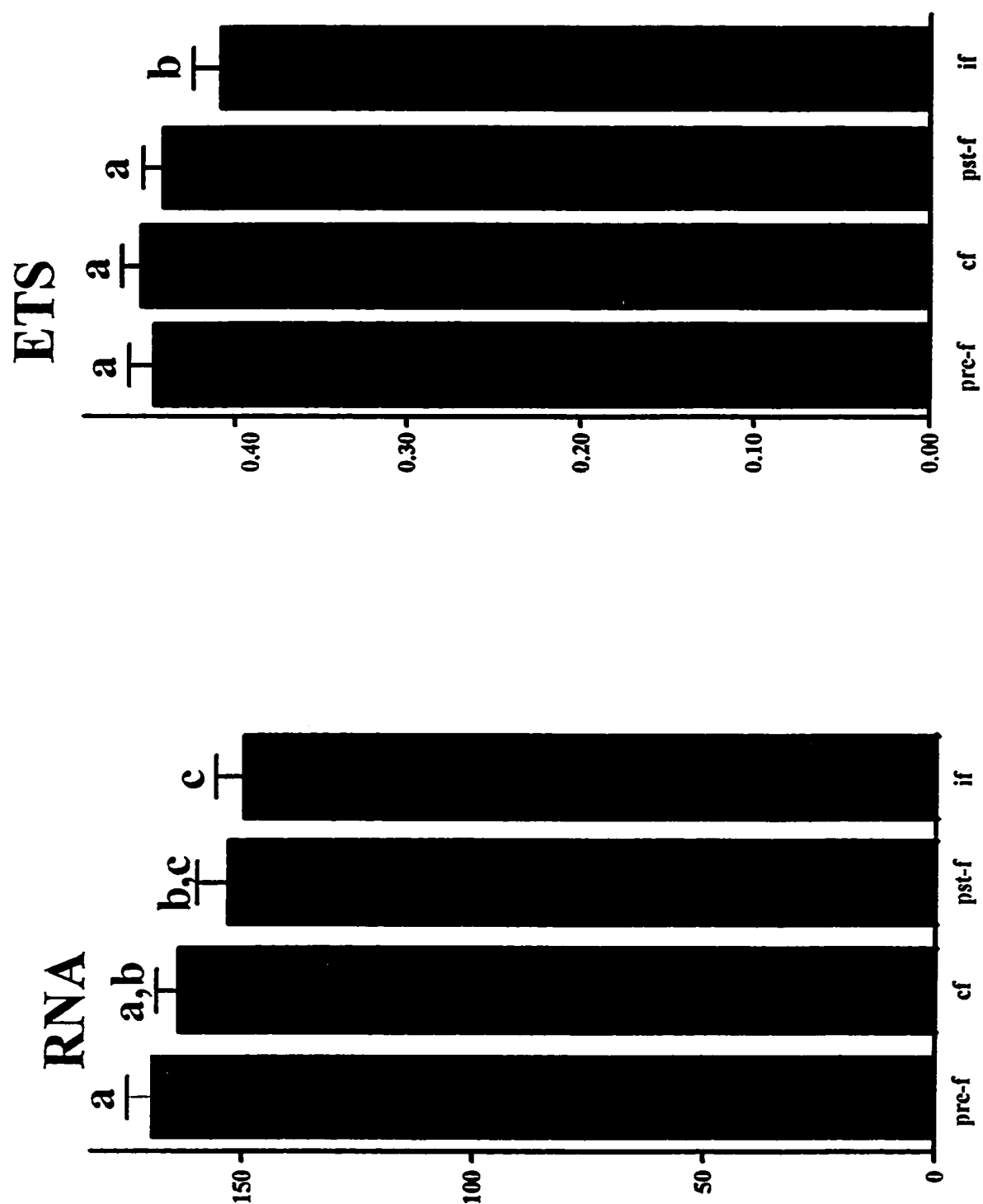


**Figure 3.14: Comparison of ETS Protein<sup>-1</sup> ( $\mu\text{l O}_2/\text{min}/\text{mg protein}$ ) and ETS DNA<sup>-1</sup> ( $\mu\text{l O}_2/\text{min}/\mu\text{g DNA}$ ) among weather classification variables. Pre-f=Pre-Frontal, CF=Cold-Front, Pst-F=Post-Front, If=Inter-Front. Error bars represent 1 standard error. Histogram bars denoted with the same letter are not significantly different (ANOVA, Tukey multiple comparison,  $\alpha=0.05$ ).**

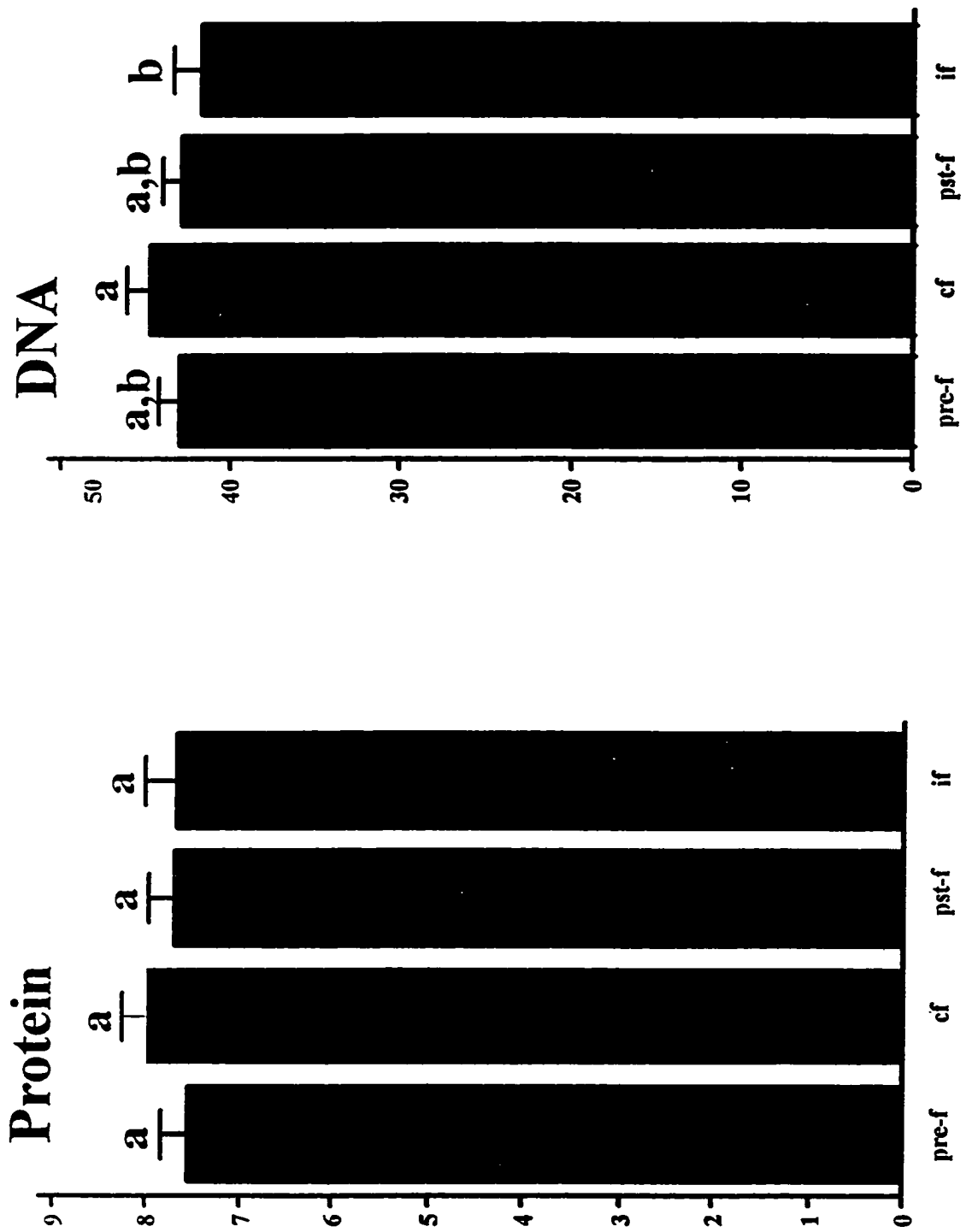




**Figure 3.15: Comparison of RNA ( $\mu\text{g}/\text{larva}$ ) and ETS activity ( $\mu\text{l O}_2/\text{min}/\text{larva}$ ) among weather classification variables. Pre-f=Pre-Frontal, CF=Cold-Front, Pst-F=Post-Front, If=Inter-Front. Error bars represent 1 standard error. Histogram bars denoted with the same letter are not significantly different (ANOVA, Tukey multiple comparison,  $\alpha=0.05$ ).**



**Figure 3.16: Comparison of DNA ( $\mu\text{g/larva}$ ) and Protein ( $\text{mg/larva}$ ) among weather classification variables. Pre-f=Pre-Frontal, CF=Cold-Front, Pst-F=Post-Front, If=Inter-Front. Error bars represent 1 standard error. Histogram bars denoted with the same letter are not significantly different (ANOVA, Tukey multiple comparison,  $\alpha=0.05$ ).**



protein and DNA content. Hence, there was no indication that younger larvae were being transported into the estuary by the winds associated with cold front passage.

### **Year to Year Comparisons**

The larvae collected during the 1993-1994 field season were significantly larger than larvae from the 1994-1995 season as measured by mg protein/larvae ( $p=0.0002$ ) but not as measured by DNA ( $p=0.52$ ). The larvae collected during the 1994-1995 season had higher condition, as indicated by the RNA content, ETS protein<sup>-1</sup> activity ( $p=0.001$ ), RNA:DNA values ( $p=0.001$ ) and RNA protein<sup>-1</sup> ratio ( $p=0.001$ ) (Table 3.5). DNA specific ETS activity was also greater in 1994-1995, although not significantly.

Surface and air temperature were significantly higher in 1994-1995 than in 1993-1994. Air and surface temperatures averaged 14.7° C and 16.2° C respectively in 1994-1995 compared to 12.6° C (air) and 14.5° C (surface) in 1993-1994. Daily mean wind speed was higher in 1993- 1994 than in 1994-1995, however the southerly component of the wind was greater in 1994-1995. There were a total of 10 distinct cold-front passages during of the two collection seasons. In 1993-1994, 21.5% of the collection days were classified as pre-frontal, compared to 46% of the collection days in 1994-1995.

### **Within Season Comparison**

Initial analysis of the seasonal variation of the condition indices showed a strong interaction between year and month among all variables, therefore each year was examined individually.

**Table 3.5: Comparison of biochemical measures of condition between field season. P-value results from ANOVA.**

	<b>Field Season</b>		
<b>Biochemical Measure</b>	<b>1993-1994</b>	<b>1994-1995</b>	<b>p-value</b>
RNA	154.68	168.42	0.0001
DNA	43.20	43.66	0.52
RNA:DNA	3.68	3.94	0.0001
ETS	0.447	0.441	0.39
ETS/Protein	0.0160	0.0175	0.0001
ETS/DNA	0.010	0.011	0.18
Protein	28.88	26.88	0.0002

### **1993-1994 Season**

The changes in biochemical measures of condition for the 1993-1994 field season are summarized in Table 3.6. The biochemical measures of larval condition in December, 1993 were significantly greater than those of larvae collected in either January or February, 1994 except for the ratio of protein to DNA (Table 3.6) . Larvae collected in December had a greater RNA:DNA value and greater RNA to protein ratio. ETS activity level, whether absolute activity or DNA or protein specific activity was significantly higher in December, 1993. Larvae collected in December, 1993 had the largest mean size based on protein and DNA content, but they were not significantly larger than the larvae from February, 1994 (in  $\mu\text{g}$  DNA/larvae) or January, 1994 (in mg protein/larvae). The larvae collected in January and February of 1994 did not differ from each other except for RNA/protein values and protein content. Larvae collected in January, 1994 had greater RNA/protein values when compared to larvae collected in February, 1994. The southerly wind component was greatest during December, 1993 but it did not differ significantly from January or February, 1994. Wind speed was greatest in January, 1994 and was significantly greater than February wind speed. Wind speed in December were equivalent to both January and February, 1994. Water temperatures were coldest in January, and warmest in February. In December, 43% of the collection days (3 of 7) were classified as pre-frontal, while only 16% (3 of 19) and 18% (2 of 11) were classified as pre-frontal periods for January and February, respectively.

**Table 3.6: Summary of biochemical measures of condition for each year, separated by month of collection. Presented are the mean value for each collection trip. Means denoted with the same letter are not different (ANOVA, Tukey multiple comparison,  $\alpha=0.05$ ).**

1993-1994		MONTH	
Biochemical Measure	December	January	February
Ratio	5.05 (A)	3.41 (B)	3.59 (B)
RNA ( $\mu\text{g/larvae}$ )	214.6 (A)	144.1 (B)	147.8 (B)
DNA ( $\mu\text{g/larvae}$ )	46.21 (A)	42.63 (B)	43.00 (A,B)
Protein (mg/larvae)	8.20 (A)	8.15 (A)	7.40 (B)
ETS ( $\mu\text{l O}_2/\text{min/larvae}$ )	0.48 (A)	0.44 (B)	0.42 (B)
ETS/DNA	0.011(A)	0.010 (B)	0.010 (B)
ETS/Protein ( $\times 1000$ )	0.062 (A)	0.057 (B)	0.056 (B)
RNA/Protein	0.029 (A)	0.021 (B)	0.018 (C)
Protein/DNA	186.77 (A)	188.02 (A)	181.76 (A)
1994-1995		MONTH	
Biochemical Measure	December	January	February
Ratio	3.48 (B)	4.33 (A)	3.66 (B)
RNA ( $\mu\text{g/larvae}$ )	127.7 (B)	182.3 (A)	179.9 (A)
DNA ( $\mu\text{g/larvae}$ )	37.41 (C)	43.06 (B)	49.97 (A)
Protein (mg/larvae)	6.69 (B)	6.84 (B)	9.18 (A)
ETS ( $\mu\text{l O}_2/\text{min/larvae}$ )	0.40 (C)	0.43 (B)	0.51 (A)
ETS/DNA	0.011(A)	0.010 (B)	0.011 (A,B)
ETS/Protein ( $\times 1000$ )	0.059 (B)	0.060 (A,B)	0.066 (A)
RNA/Protein	0.019 (C)	0.029 (A)	0.022 (B)
Protein/DNA	195.02 (A)	161.41 (B)	196.03 (A)



### **1994-1995 Season**

The changes in biochemical measures of condition for the 1994-1995 field season are summarized in Table 3.6. The highest RNA:DNA and RNA/ protein values occurred in January, 1995 at which time values for both indices were greater than during either of the other two collection trips. For RNA:DNA, February, 1995 did not differ significantly from December, 1994, but RNA/protein values were greater in February, 1995. Measures of ETS activity were greatest in February, 1995 for total ETS activity and ETS/protein. ETS/DNA was higher in December, 1994 than in February, 1995, but the difference was not significant. In terms of protein, the larvae captured in February, 1995 were 26% larger than larvae caught in the previous two months. In terms of DNA, February larvae were 20% larger. As in the 1993-1994 season, the southerly wind component was greatest during the month of highest RNA based condition indices but the difference among months was not significant. Mean wind speed was greatest during the month of February, this was also the month of coldest air and surface temperature. Both air and water temperature were significantly higher in December, 1994. In December, 1994, 42% (5 of 12) of the collection days were pre-frontal, in January, 1995 57% (8 of 14) and February 33.3% (3 of 9) of the collection days were pre-frontal.

## **DISCUSSION**

### **Methodological Considerations**

The handling procedure used in photographing the larvae for a concurrent study had no adverse effect on the measurement of the biochemical indicators of condition.

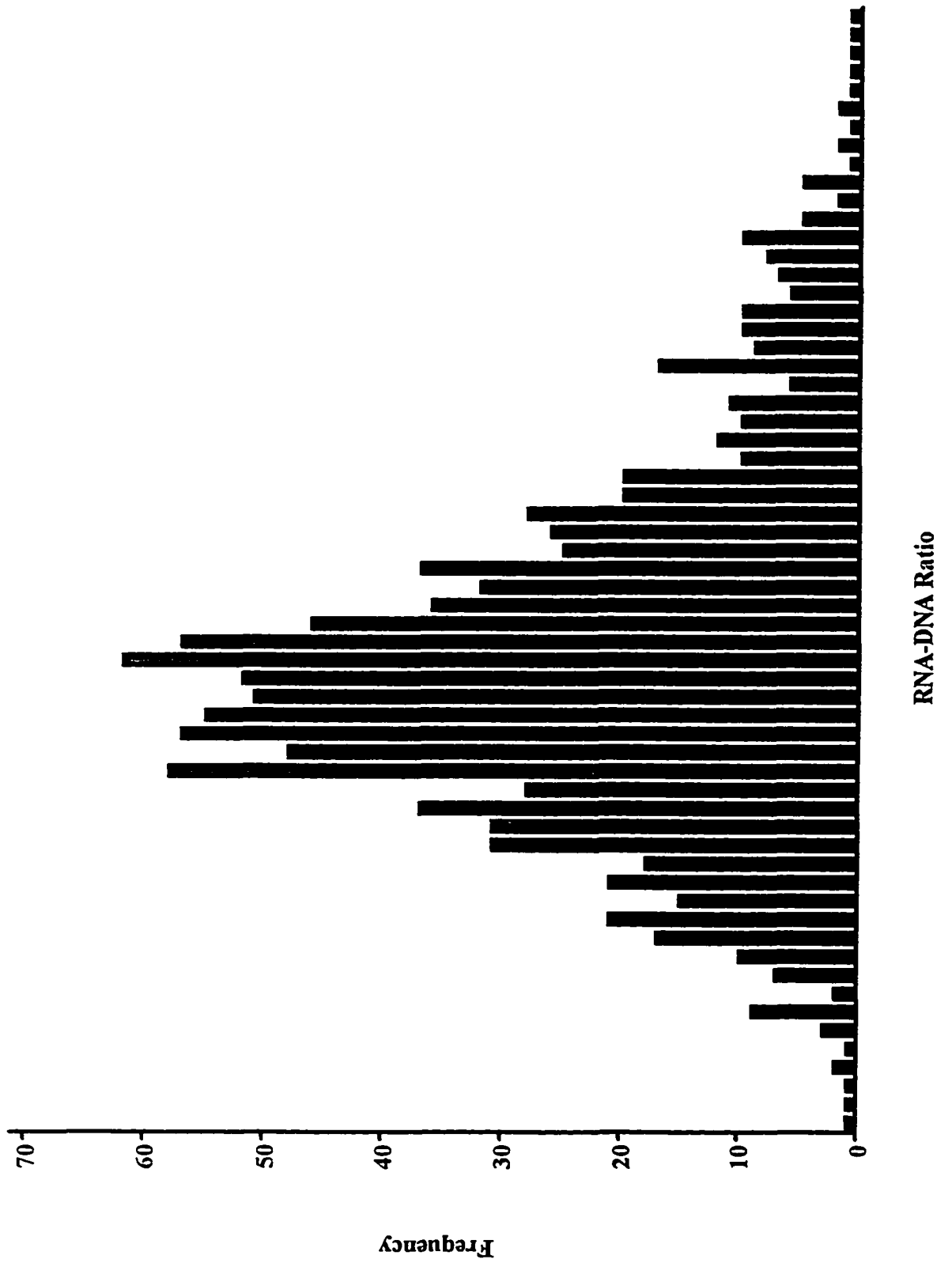
Chilling the larvae allowed for additional information to be gathered and may be used as a way to reduce larval activity levels without killing them. Mortality prior to liquid nitrogen preservation may have an adverse effect on the biochemical measures of condition (Ferron and Leggett, 1993).

The water temperature at the time of collection did not have an effect on the activation energy of the ETS enzymes of the larvae. The Arrhenius activation energies,  $E_a$ , for larvae reared at different temperatures were not statistically different, indicating that the enzyme-activation energy is independent of *in situ* temperatures; thus, activity measured at a constant temperature can provide an estimate of enzyme concentrations. Additionally, the  $E_a$  calculated in this study (17.1-20.4 kcal mol<sup>-1</sup>) correspond well with published values for micro- and zooplankton (11.7 -21.9 kcal mol<sup>-1</sup>) (Packard et al., 1975) as well as those calculated for leptocephali (11.0-15.7 kcal mol<sup>-1</sup>) (Pfeiler and Govoni, 1993).

### **Overall Condition**

The larvae collected over the two spawning seasons were healthy, with very few individuals showing signs of starvation. The critical RNA:DNA ratio suggested by Robinson and Ware (1988) of ~2 was present in less than 3% of the larvae. There was also a bell-shaped distribution of RNA:DNA ratios (Figure 3.17), indicating that the population is not truncated at the low end of the observed ratios, which would suggest the loss of starved or low-condition larvae (Robinson and Ware, 1988). In general, the observed values of the biochemical measure for menhaden larvae correspond well to the

**Figure 3.17: Frequency histogram of RNA-DNA ratios of all larvae collected. Horizontal axis ranges from 1.0 to 7.7. Individual bars represent groupings based on a 0.10 range of RNA:DNA values.**



literature reported values for other species. RNA:DNA values averaged 3.82 and correspond well with reported values for field collected larvae (Canino et al., 1991; Setzler-Hamilton et al., 1987) and well-fed laboratory-reared species (Westerman and Holt, 1994; Wright and Martin, 1985). But direct comparisons of nucleic acid concentrations should be viewed with caution because specific assay conditions may affect the values making inter-study comparisons tenuous (Canino and Caldarone, 1995). The RNA protein<sup>-1</sup> values ( $\bar{x}$ =22.88) for the menhaden larvae are approximately 50% of those reported for laboratory reared herring larvae, *Clupea harengus* (Hoilhan et al., 1995) but are similar to those of juvenile cod, *Gadus morhua*, tissues (Foster et al., 1993), but again; differences in techniques should be considered while making comparisons. The ETS activity levels for the menhaden larvae were 0.446  $\mu$ l O<sub>2</sub>/min/larva (26.7  $\mu$ l O<sub>2</sub>/hr/larvae), the only other published reports of ETS activity in fish larvae are Yamashita and Bailey, 1990. The pollock (*Theragra chalcogramma*) larvae used in by Yamashita and Bailey (1990) were only as large as 8.3 mm, the smallest menhaden in this study was for which length was measured was 13.6 mm. Using the regression equation for ETS activity and length generated in this study, an 8.3 mm menhaden larvae would have an ETS activity level of 2.0  $\mu$ l O<sub>2</sub>/hr/larva, which corresponds reasonably well with the 3.5  $\mu$ l O<sub>2</sub>/hr/larva reported for 8.3 mm pollock.

#### **ETS Activity as a Condition Index**

One of the objectives of this study was to examine the inter-relationships of the various biochemical measures of condition. Of these measures, RNA:DNA and RNA protein<sup>-1</sup> have been extensively tested under laboratory setting and have been shown to

accurately reflect the recent feeding success of fish larvae (Buckley, 1982; Clemmesen, 1988, 1989; Westerman and Holt, 1994; Mathers et al., 1993; Houlihan et al., 1995). The results presented here show a strong correlation between the two RNA-based indices measures. ETS activity as a measure of condition is relatively new to the fish literature and laboratory calibration of its potential as an indicator of condition in fish larvae is forthcoming (Dortch, in prep). However, the relationships presented here between ETS activity-based condition and the more documented RNA-based indices are interesting. The strongest correlation between ETS activity and RNA-based condition occurred when ETS activity was normalized to protein content, rather than DNA content. While ETS activity corrected for DNA is attractive in that it provides an estimate of per-cell metabolic activity, it may not accurately reflect the overall condition of the larvae. Normalizing the enzyme activity to protein may be a superior indicator of condition (when compared to ETS DNA<sup>-1</sup>) in that ETS protein<sup>-1</sup> relates more strongly with protein synthesis rates than with cellular growth.

#### Size Dependence of Condition Indices

RNA:DNA values have been shown to be age and size dependent in several species, including winter flounder, *Pseudopleuronectes americanus*, (Buckley, 1982), Striped jack, *Caranx delicatissimus* (Takii et al., 1994), red drum, *Sciaenops ocellatus* (Westerman and Holt, 1994) and herring, *Clupea harengus*, (Folkvord et al., 1996). In these studies the RNA:DNA increased with larval age within each feeding regime. This necessitates the consideration of age or size of the larvae in the analysis of condition, especially in young larvae. In older larvae, the relationship between age and RNA:DNA

is less pronounced (Buckley, 1982; Takii et al., 1994; Folkvord et al., 1996). Richard et al. (1991) found that older larvae had a reduced response in terms of RNA:DNA to period of starvation. While the starvation interval was still reflected in the ratio, the magnitude of the response was age dependent. In this study, the result of a weak correlation between RNA:DNA condition indices and size is most likely a reflection of the fact that the menhaden were late-stage larvae and the ratios observed are more of a reflection of the condition, rather than the age of the larvae.

Data on the size-specific response of RNA:protein is limited. Mathers et al. (1993) examined the RNA:protein values in *Oncorhynchus mykiss*. Although not addressed in their study, there appears to be no trend in RNA:protein values in relation to age. Houlihan et al. (1995) reported no significant difference in RNA:protein values among herring larvae (*C. harengus*) ages 7-17 d. The negative correlation of RNA:protein observed in this study is an indication that there may be a size-specific dependence of this condition index for older larvae, but controlled laboratory work is needed to fully understand the relationship between size and RNA protein<sup>-1</sup>.

Uncorrected ETS activity, as well as activity of other metabolic enzymes (Clarke et al., 1992), increases rapidly with size (Yamashita and Bailey, 1990; G-Toth et al., 1995; Savenkoff et al., 1995). In the literature, the relationship between size and ETS activity has been reported as linear, exponential and logarithmic functions. Savenkoff et al. (1995) suggest that the logarithmic function is most appropriate for ETS activity. A logarithmic model was used for the present study, but as in Savenkoff et al. (1995) observed, the logarithmic model did not greatly improve the explanatory power ( $r^2$ ) over

the linear or exponential fits. The cause of the lack of improvement in the choice of model is probably two fold. First, there was a somewhat limited size class of larvae, therefore only a portion of the total logarithmic function of ETS activity and length was observed. Second, larval condition in this study was variable and the strong linear relationships of controlled laboratory studies is clouded by the environmentally induced noise in the measurements of field caught larvae.

Size-corrected ETS activity showed a non-significant relationship to larval length. This may indicate that protein-specific ETS activity is a robust estimator of condition that is age and size independent. In an analysis of red drum (*Sciaenops ocellatus*) larvae (0 to 15 d. old), Clarke et al. (1992) found that protein-specific lactate dehydrogenase and protein-specific citrate synthase activities showed no clear pattern with age or development, but the metabolic enzymes were useful as estimators of larval condition. Pfeiler and Govoni (1993) reported that the weight-specific ETS activity in various species of leptocephalus larvae decreased with length of the larvae, but given the uniqueness of the Elopomorph life cycle, this pattern can not be assumed to be universal in larval development. Clearly, more work needs to be accomplished concerning the tendencies of protein-specific enzyme activity in relation to size and age of fish larvae. However, based on the results of this study and those of Clarke et al. (1992), metabolic enzyme activity levels may be shown to be age- and size-independent estimators of condition, thus eliminating the need for analysis based upon grouping of specimens.



### **Correlation Between Environmental Variables And Condition Indices**

The lack of any strong correlation between the condition indices and the environmental variables was unexpected. There are numerous reports on the effects of temperature on the RNA:DNA ratios (Buckley, 1982; Buckley et al. 1990; Malloy and Targett, 1994) and protein synthesis (Mathers et al. 1993). The explanation for this may be that the highly dynamical environmental conditions along the Louisiana coast during the winter do not allow a temperature-dependent response to be manifested. To my knowledge, there have been no reports on the latency of the response of biochemical condition indices to changes in temperature. The latency of responses to feeding changes is generally 1-2 d depending on the rearing temperature (Ferron and Leggett, 1994). It seems reasonable that since the magnitude of RNA:DNA response to feeding ration is greater than the response to rearing temperature (Malloy and Targett, 1994) that the latency of the response to a temperature change would be greater than 1-2 d. It is apparent from the examination of Figure 3.2 that the temperature regime rarely maintains itself for periods longer than 1-2 days. The lack of a strong correlation between the southerly wind component and the condition indices is in contrast to the significant effect of the southerly wind dominated pre-frontal classification. But, the weather classification scheme used in this study is effectively a filter, eliminating the slight southerly wind components from the other weather classifications.

### **Inter-annual Variation**

The examination of the condition of menhaden larvae in relation to the progression of the spawning season exhibited no consistent pattern between the two

years examined, indicating that mechanisms other than the progression of time are influencing the observed variation in condition. Govoni et al. (1983) found that the diet of menhaden larvae collected in the northern Gulf of Mexico have a seasonal component. The authors analyzed the gut contents of menhaden larvae in December and February of 1979-80 and 1980-81. Over all size classes, larvae tended to have a more diverse diet in February than in December. But for larger larvae (15-20mm), the differences were less pronounced with only February, 1981 differing from the other 3 months examined. Weather data is unavailable, but perhaps in addition to increasing the condition of the larvae through increased encounter rates, atmospheric frontal passage may also alter the prey field of the larvae. The unidentified chyme, which was predominate in the menhaden guts, may be detritus flushed out of the estuaries by the northerly winds.

### **Effect of Frontal Passage on Condition Indices**

The most interesting result in this study is the co-incidence of high condition with the southerly wind dominated pre-frontal periods. In an overall comparison, RNA:DNA values, RNA:protein, protein specific and DNA specific ETS activity were all highest during the prefrontal period. In the comparison between years, the larvae collected in 1994-1995 had significantly higher condition in terms of protein-specific ETS activity, RNA:DNA, RNA:protein and overall RNA content than the larvae collected in 1993-1994. There were twice as many pre-front days in 1994-1995 than in 1993-1994. Within each year, larvae had the highest condition in terms of RNA:DNA and RNA:protein during the months with the greatest frequency of pre-frontal days. The results presented here are in direct contrast to the results of Maillet and Checkley (1991)

and the stable ocean hypothesis. Higher condition larvae in association with the southerly winds suggests that the menhaden larvae may be benefitting from the wind-induced turbulence and subsequent increases in prey-encounter rates as suggested by Rothschild and Osborn (1988) and MacKenzie et al. (1994).

In this study there is no indication of the dome-shaped relationship between the magnitude of the wind and increased ingestion proposed by MacKenzie et al. (1994) (Figure 3.11-3.12). The model of MacKenzie et al. (1991) suggest that the maximum increase in ingestion rate should occur at wind speeds of 15-20 m s<sup>-1</sup>. The winds associated with the cold front passages in this study averaged 5 m s<sup>-1</sup>. However, limitations in the model (homogenous prey distribution in a closed system, limits on turbulent transport of predator and prey) may result in an over estimation of the winds associated maximum ingestion, and may explain the discrepancy between the model and other field studies (Dower et al., 1996). Conversely, the slight positive correlation between the southerly wind component and RNA:DNA may represent the lefthand side of the dome relationship and wind speeds did not reach levels that result in the advection of prey beyond the pursuit capabilities of the larvae (MacKenzie et al., 1994). Advection beyond the larval pursuit capabilities is the mechanism which results in the downward trend of the right-hand portion of the domed relationship.

The duration of the southerly winds associated with the pre-frontal period are usually greater than 48 h, thus given a latency of biochemical measures of condition of 1 day, the greatest condition should be co-incident with the southerly winds rather than lagged. Additional evidence for pre-frontal association is the days following the pre-

frontal winds, classified as cold-front, are exclusively ranked below only pre-frontal in terms of condition. Thus, as the winds shift to the north as the cold front passes the coastline, the reduction in wind-generated turbulence (less fetch) reduces encounter rate and condition indices begin to decline.

## **CHAPTER 4: MORPHOMETRIC ANALYSIS OF CONDITION**

### **INTRODUCTION**

Morphometrics have been used to classify the condition of fish larvae since the 1950's (Ferron and Leggett, 1994). In early larval development, growth is rapid and morphological development is complex. Since growth and morphological development are coupled, it seems reasonable that changes in growth rate due to differential food availability would be reflected in the morphological shape of the larvae. Traditionally, morphometric condition indices have relied on ratios of two linear measurements or a related form of statistical analysis. For example, Theilacker (1986) used stepwise-discriminate analysis, based on 11 body-part measurements, and found the predictive capabilities of that analysis to be the same as a more laborious histological analysis. Koslow et al. (1985) also used stepwise-discriminate analysis but concluded that the bivariate ratios provided just as much descriptive power as the stepwise discriminate analysis. Fulton's K, defined as the ratio  $\text{weight}/\text{length}^3$ , was used by Frank and McRuer (1989) to identify higher condition haddock (*Melanogrammus aeglefinus*) larvae in well mixed areas off Nova Scotia. Fulton's K may be the most commonly used morphometric condition index, particularly in larger fish (Bolger and Connolly 1989). Powell et al. (1990) used the relationship between 6 linear measurements to characterize the condition of spot (*Leiostomus xanthurus*) larvae in the Gulf of Mexico, and Burke et al. (1995) used the amount of variability in the regression of two different length measurements to classify Atlantic croaker (*Micropogonias undulatus*) larvae captured along a pollution gradient in Galveston Bay, Texas.

All of the common morphometric analyses of condition have relied on the measurement of variables thought to be sensitive to food derivation (body depth, weight) in relation to variables presumed to be resistant to starvation (eye diameter, head length, total length). There is often a size dependency on the condition measures, limiting the valid analysis to specific size classes of individuals (Cone, 1989). Also when examining distance measures, all the information regarding the orientation and the spatial relationships among the measurements is ignored. Bookstein (1991) introduced a multivariate technique (called the "thin-plate spline") that utilizes a collection of landmark coordinate measurements to characterize the overall shape of a specimen independently of organism size. Additionally, the use of landmarks eliminates the need of *a priori* knowledge of the variables to be measured (Horn, 1996). This technique has allowed for the quantification of subtle shape differences in the faces of children due to pre-natal chemical exposure, previously diagnosed qualitatively as "the face" (Bookstein, 1991). The method has also been widely employed as a technique to classify closely related species in taxonomy (Rohlf, 1991). Recently, Horn (1996) used the thin-plate spline technique to measure shape change in laboratory reared larval razorback suckers (*Xyrauchen texanus*) fed differing rations.

Bookstein's (1991) thin-plate spline is an interpolating function that minimizes the energy required to bend a plate of material between known  $X$ ,  $Y$  and  $Z$  coordinate values. Bookstein (1991) realized that the orthogonality of the  $Z$  direction is not computationally essential for the use of the spline, and that the same function could be used to examine horizontal displacements in landmark position relative to those of a reference specimen.

Landmarks in this method of shape analysis are the  $X$  and  $Y$  Cartesian coordinates of consistently identifiable points among a series of images or specimens. The location of these landmarks in relation to those of a consensus or reference organism is examined for patterns relating to the biological question at hand, in this study, the condition of the fish.

The objective of this portion of the study was to determine if larval shape varied in relation to the biochemical measures of condition. It was hoped that this method of shape analysis would provide a rapid and cost-effective alternative to the biochemical indicators of condition.

## **MATERIAL AND METHODS**

Menhaden larvae were collected from Port Fourchon, Louisiana as described in the previous chapter. After capture, a lateral view photograph of the larvae was taken using a Nikon F-3 camera, using T-Max 100 black and white film, mounted on a Wild dissecting scope. The film was processed to negatives and the images scanned into a computer using a digital scanning camera. The scanned images were enhanced and landmark coordinates were digitized using software written with the Precision Visuals Workstation and Visualization Environment (PV-WAVE) application. Image resolution was approximately 0.02 mm of the fish's body per image pixel. Initially, a series of 72 landmarks were identified from generalized larval characteristics (insertion of fins, anal vent, etc.) and from lines dorsal-ventrally bisecting the larva at 4 equidistant locations along the body axis. At each point of bisection, landmarks were recorded at the dorsal margin of the gut (if present), dorsal margin of the notochord and dorsal and ventral body margins. For practical reasons, only 42 individual landmarks were consistently

identifiable in the photographs (Table 4.1, Figure 4.1). Several of the consistently identifiable landmarks (15 of 42) were correlated in that they were located along a line of bisection, thus only represented variation in the  $Y$  direction.

### Mathematical Computations

The computation of the thin-plate spline relative warp analysis is briefly described as follows (Bookstein, 1991, Rohlf, 1992). In this example, a series of  $p$  landmarks are recorded on a single specimen. A  $2 \times p+3$  matrix of the  $X, Y$  landmark coordinates of this specimen ( $Y$ ) can be transformed to the landmark coordinates of the reference organisms while minimizing the “bending energy” using the following (matrix) equation:

$$Y = N L \quad (1)$$

The  $N$  ( $2 \times p+3$ ) matrix contains the spline coefficients for this particular specimen, and the  $L$  matrix is composed of three sub-matrices: The  $U(r)$  function values defined below ( $p \times p$ , upper left), a column or row of ones concatenated to the reference specimen coordinates ( $3 \times p$ , right 3 columns and bottom three rows), and a matrix of zeros ( $3 \times 3$ , bottom right). The  $U(r)$  function minimizes the “bending energy” necessary to transform the specimen to the reference specimen, and is defined as:

$$U(r) = r_{ij}^2 \text{Log}_e(r_{ij}^2)$$

where  $r_{ij}$  is the distance between the paired  $i^{\text{th}}$  and  $j^{\text{th}}$  landmark of the reference organism.

Equation (1) is then solved for  $N$ :

$$Y L^{-1} = N \quad (2)$$



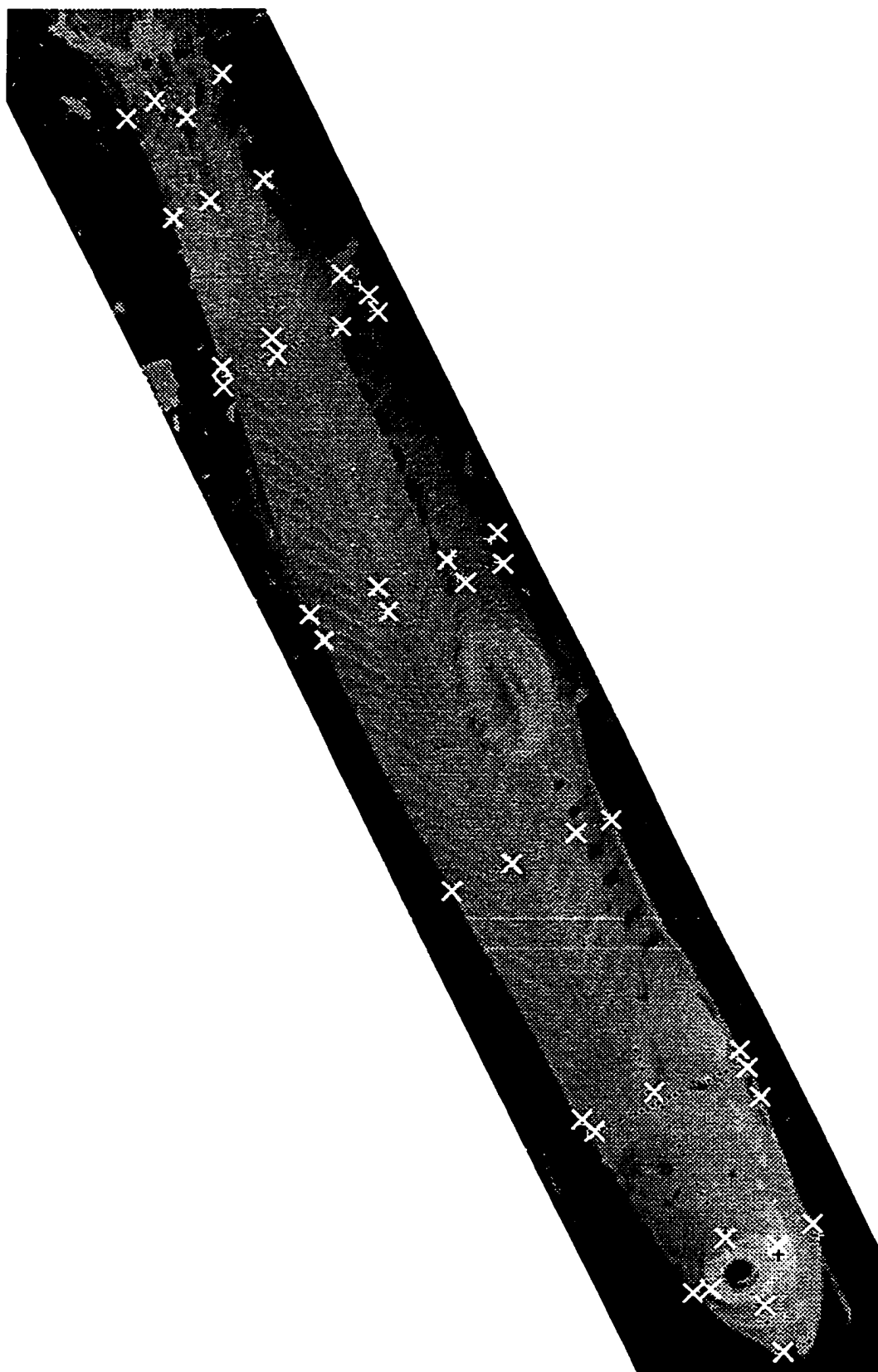
**Table 4.1: List of landmarks identified and recorded on menhaden larvae. Line 1-4 are equidistant vertical lines drawn perpendicular to the body axis between the two baseline landmarks (Tip of snout and urostyle tip). Line 5 was drawn perpendicular to the body axis at the urostyle tip. At 5 landmark locations a vertical line perpendicular to the body axis was drawn to facilitate the location of the subsequent landmarks (denoted ->).**

<b>LANDMARK</b>	<b>LINE PERPENDICULAR TO BODY AXIS</b>
Tip of snout (upper jaw, premaxilla)	
Eye, anterior margin	
Eye, posterior margin	
Eye, ventral margin	Yes
Eye, -> dorsal margin of eye	
Eye, -> dorsal body margin intersection	
Eye, -> ventral body margin intersection	
Line 1 -> ventral margin of body	
Line 1 -> dorsal margin of body	
Operculum, ventral margin of body	
Operculum, posterior margin	Yes
Operculum, ->ventral body margin intersection	
Operculum, ->dorsal body margin intersection	
Line 2 -> ventral margin of body	
Line 2 -> dorsal of gut	
Line 2 -> dorsal of notochord	
Line 2 -> dorsal margin of body	
Line 3 -> ventral margin of body	
Line 3 -> dorsal of gut	

(Tab. con'd.)

Line 3 -> dorsal of notochord	
Line 3 ->dorsal margin of body	
Dorsal fin anterior insertion	Yes
Dorsal fin ant. ins->ventral body margin	
Dorsal fin ant. ins-> dorsal of gut	
Dorsal fin ant. ins-> dorsal notochord	
Line 4 -> ventral margin of body	
Line 4 -> dorsal of gut	
Line 4 -> dorsal of notochord	
Line 4 -> dorsal margin of body	
Anus	Yes
Anus -> dorsal of notochord	
Anus -> dorsal body margin intersection	
Anal fin anterior insertion	
Anal fin posterior insertion	Yes
Anal fin post. ins. -> dorsal of notochord	
Anal fin post. ins. -> dorsal margin of body	
Notochord at flexion ventral margin	
CENTER OF NOTOCHORD AT FLEXION	
Notochord at flexion dorsal margin	
Urosytle tip	Yes
Line 5 -> Caudal peduncle ventral margin	
Line 5 -> Caudal peduncle dorsal margin	

**Figure 4.1: Image of a typical larval gulf menhaden with representative positions of the landmarks.**



The first  $p$  rows and columns of  $L^{-1}$  are called the bending energy matrix,  $L^{-1}_p$ .  $L^{-1}_p$  is decomposed into eigenvalues and eigenvectors ( $L^{-1}_p = E \Lambda E^T$ ). The eigenvectors ( $E$ ) are termed the principal warps, and can be thought of as modes of potential landmark displacement. There are  $p$  principal warps, one for each landmark coordinate pair. The principal warps represent a "reference specimen"-based coordinate system, within which all other specimens can be positioned with respect to the reference specimen. The reference specimen itself can be thought of as located at the origin formed by the intersection of all the principal warps. The eigenvalues ( $\Lambda$ ) are an indicator of the bending energy, and are inversely related to scale. Large eigenvalues correspond to deformations over short distances (it takes more energy to bend a sheet of metal between two points that are close together). Three eigenvalues will be zero, corresponding to the affine or uniform shape change across all specimens, and are dropped from further analysis ( $\Lambda$  becomes  $(p-3 \times p-3)$  and  $E$  becomes  $(p \times p-3)$ ). Subsequent to the decomposition, equation (2) becomes:

$$Y E \Lambda E^T = N \quad (3)$$

Now assume that the  $Y$  matrix contains the  $X, Y$  coordinates for all the specimens in the sample, standardized to a baseline (Bookstein Shape Coordinates; Bookstein, 1991).

The product  $(Y E)$  transforms the sample specimens from the landmark based coordinate space to the principal warp space, which was determined by the reference specimen. The principal warps can be weighted by a scaling factor ( $\Lambda^{\alpha/2}$ ) associated with the bending energy. If  $\alpha=0$ , all principal warps are weighted equally, while setting  $\alpha=1$  weights the

principal warps by the inverse square root of the bending energy (or eigenvalues).

Hence the projection of the sample in to principal warp space becomes:

$$\mathbf{Y} \mathbf{E} \Lambda^{\alpha/2} = \mathbf{W} \quad (4)$$

### **Relative Warps**

If  $\alpha=0$ , the calculation of the relative warps is equivalent to principal component analysis, with the relative warps corresponding to principal component axes. Singular value decomposition of the  $\mathbf{W}$  matrix yields:

$$\mathbf{W} = \mathbf{S} \mathbf{D} \mathbf{R}^T$$

where  $\mathbf{R}$  is the matrix of relative warps that describe the axes of maximum variance.

These are equivalent to the principal components when  $\alpha=0$ .  $\mathbf{D}$  is a diagonal matrix of singular values, and  $\mathbf{S}$  is the matrix of sample specimen relative warp scores (principal component scores if  $\alpha=0$ ).

### **Interpretation of Thin-Plate Spline Relative Warp Analysis**

The thin-plate spline coefficients,  $\mathbf{N}$ , of each  $X$  or  $Y$  coordinate are the coefficients used to transform the reference organism into each individual sample specimen. These coefficients can be compared to independent variables of interest, in this study biochemical indicators of condition. As in principal component analysis, the scaled relative warp scores ( $\mathbf{S} \mathbf{D}$ ) can be plotted against each other to provide an ordination of the specimens based on the shape. The ordination can then be examined for patterns relating to the biological question at hand.

All analyses were conducted using programs written in SAS 6.12. For the computation of Bookstein Shape Coordinates (Bookstein, 1991), the upper tip of the

jaw and the posterior tip of the notochord (prior to the flexion) served as the anterior and posterior baseline landmark, respectively. Since there was no *a priori* reason to suspect shape change to be related to the bending energy,  $\alpha$  was set to zero for all analyses. The mean landmark location of all specimens was used as a reference organism. For ordination of specimens based on scaled relative warp scores and biochemical indices, the samples were categorized based on the quartiles of each biochemical condition index. For the comparison of the biochemical and morphometric techniques, only RNA:DNA, RNA protein<sup>-1</sup>, ETS protein<sup>-1</sup> and ETS DNA<sup>-1</sup> were used in the analysis.

## RESULTS

A total of 104 larvae were examined using thin plate spline relative warp analysis. The larvae ranged in length from 13.6 mm to 22.4 mm ( $\bar{x}$ =19.74 mm). The range of biochemical indices encompassed the entire range of all larvae collected though out the entire study (Table 4.2).

A correlation analysis between measures of biochemical condition and spline coefficients (N) was run using the entire data set (excluding baseline coordinate pairs) to identify the most promising landmarks for detailed examination and analysis. None of the spline coefficients were significantly related to more than any two of the biochemical measures of condition. Of the significant correlations, only two had correlation coefficients larger than  $r=0.35$ , eye anterior margin and line 2, dorsal body margin (Figure 4.2-4.4). A correlation between the spline coefficients and the biochemical condition indices would indicate that the displacement of a landmark coordinate from the

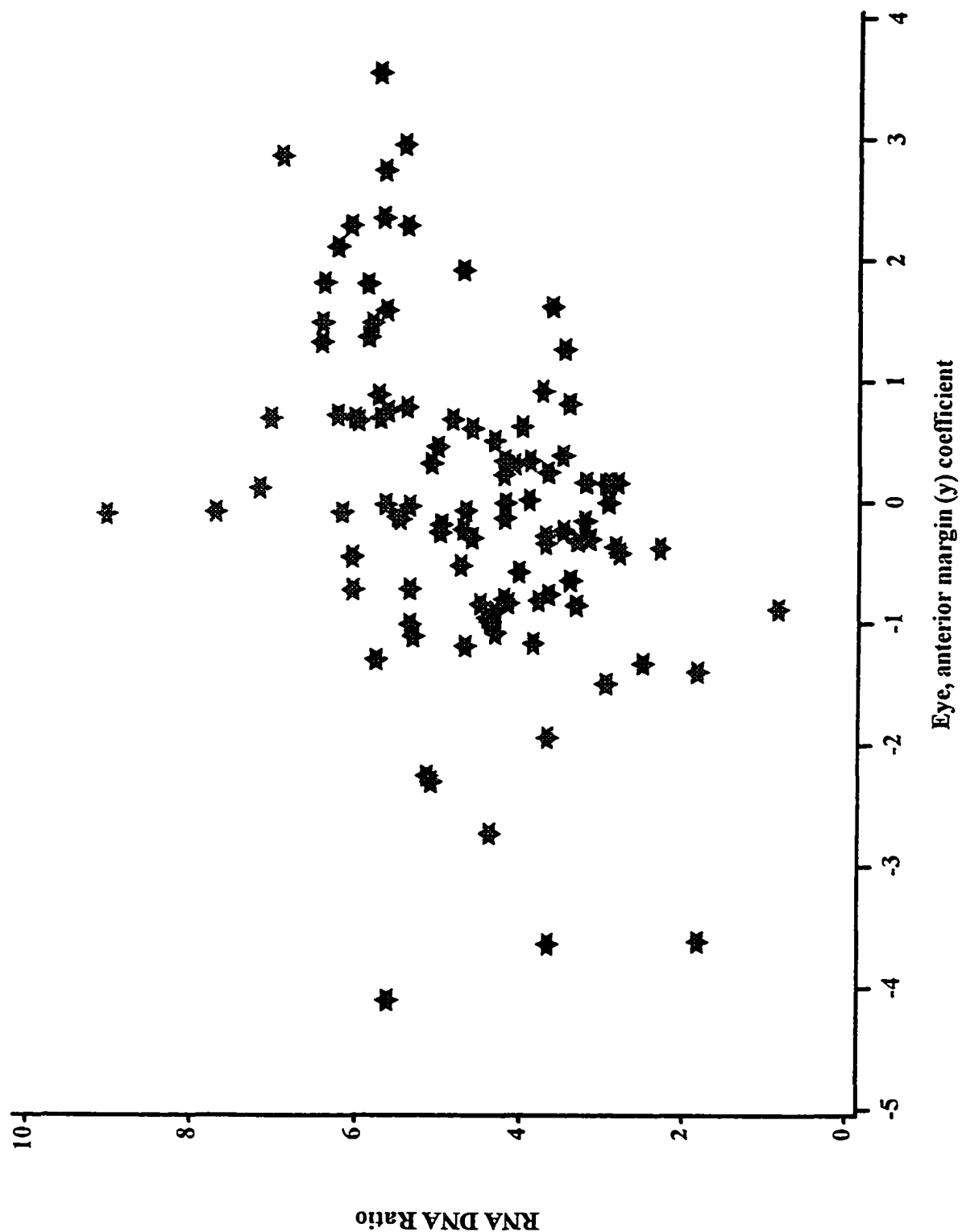
**Table 4.2: Summary statistics of the biochemical measures for menhaden larvae used in the morphometric analysis.**

Biochemical Measure	n	Mean	Range	Standard Error
RNA:DNA	104	4.61	1.82 - 9.02	0.134
RNA Protein <sup>-1</sup> ( $\mu\text{g RNA/mg protein}$ )	102	26.82	6.31 - 59.84	1.02
ETS DNA <sup>-1</sup> ( $\mu\text{l O}_2/\text{min}/\mu\text{g DNA}$ )	104	0.011	0.005 - 0.023	0.0003
ETS Protein <sup>-1</sup> ( $\mu\text{l O}_2/\text{min}/\text{mg protein}$ )	102	0.064	0.030 - 0.121	0.0015
Length (mm)	114	19.74	13.66 - 22.34	0.14

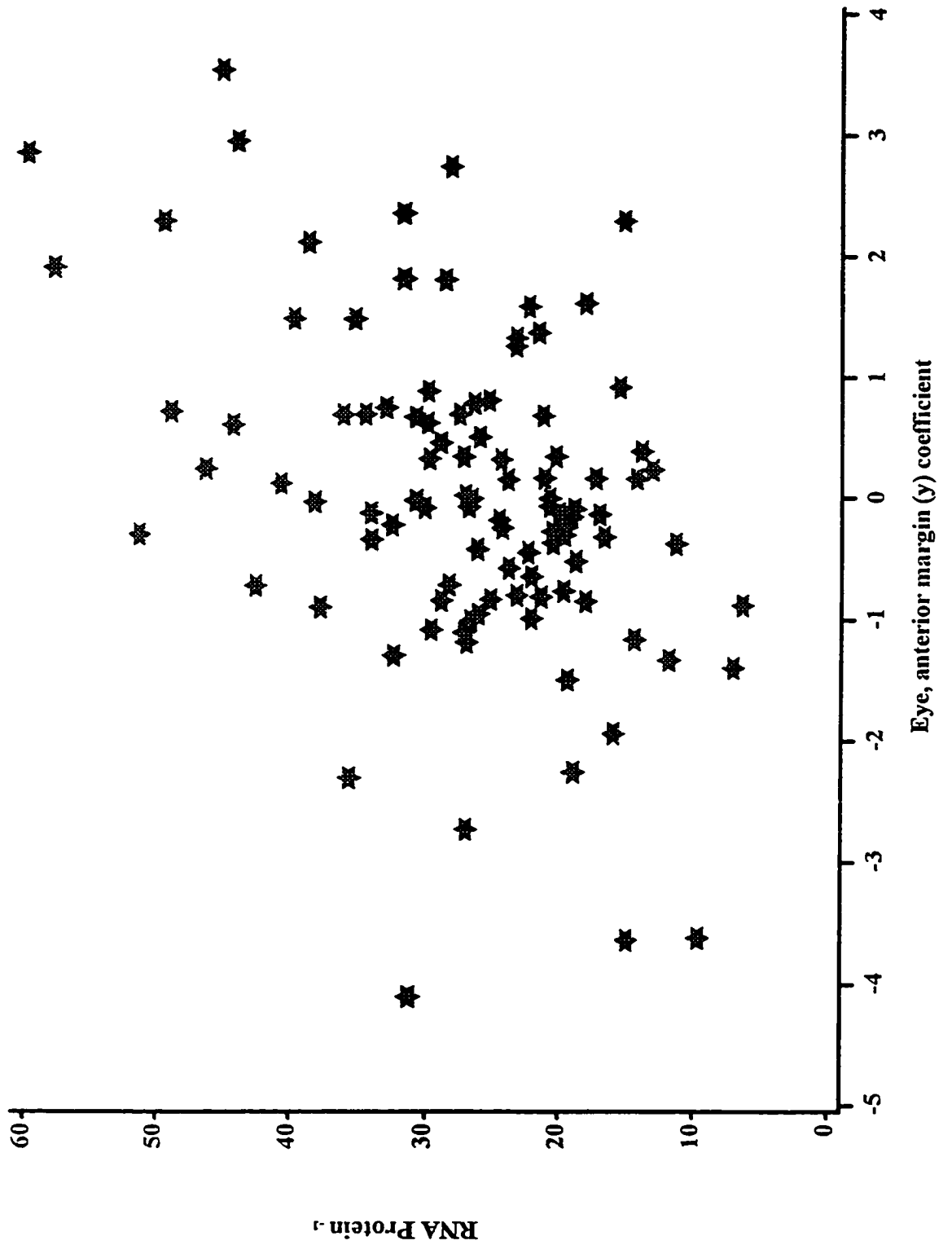


**Figure 4.2: Spline coefficient for the y coordinate of the landmark located at the anterior margin of the eye plotted against RNA-DNA values.**

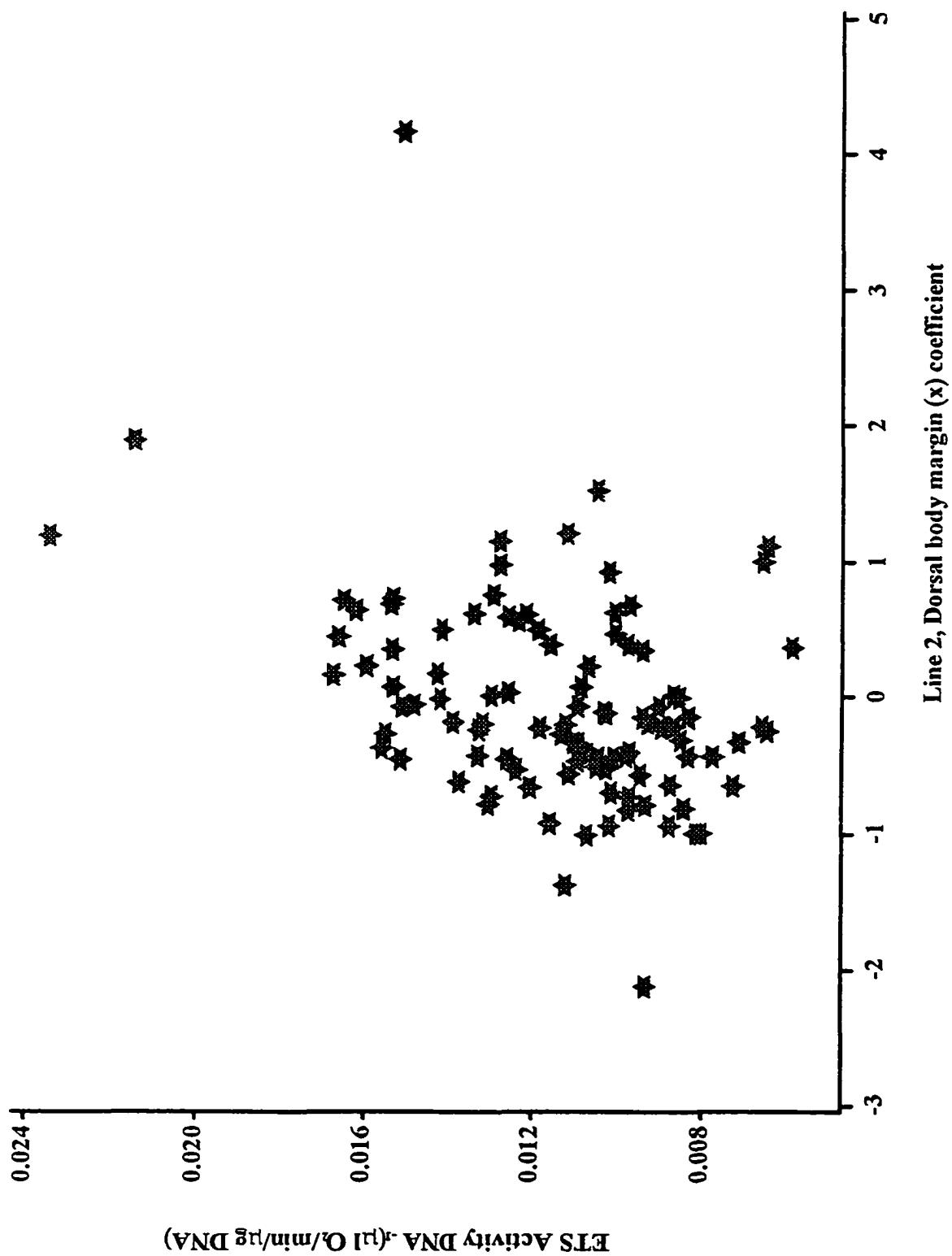
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**Figure 4.3: Spline coefficient for the y coordinate of the landmark located at the anterior margin of the eye plotted against RNA protein<sup>-1</sup> values.**



**Figure 4.4: Spline coefficient for the y coordinate of the landmark located at line 2, dorsal margin of the body plotted against ETS DNA<sup>-1</sup> values.**



reference organism to the samples was related to the relative health of the larvae. The failure to find any strong correlation between the spline coefficients and the measures of condition indicates that the landmark location does not reflect the overall condition of the larvae. The ability to predict larval condition based on the values of the spline coefficients is low.

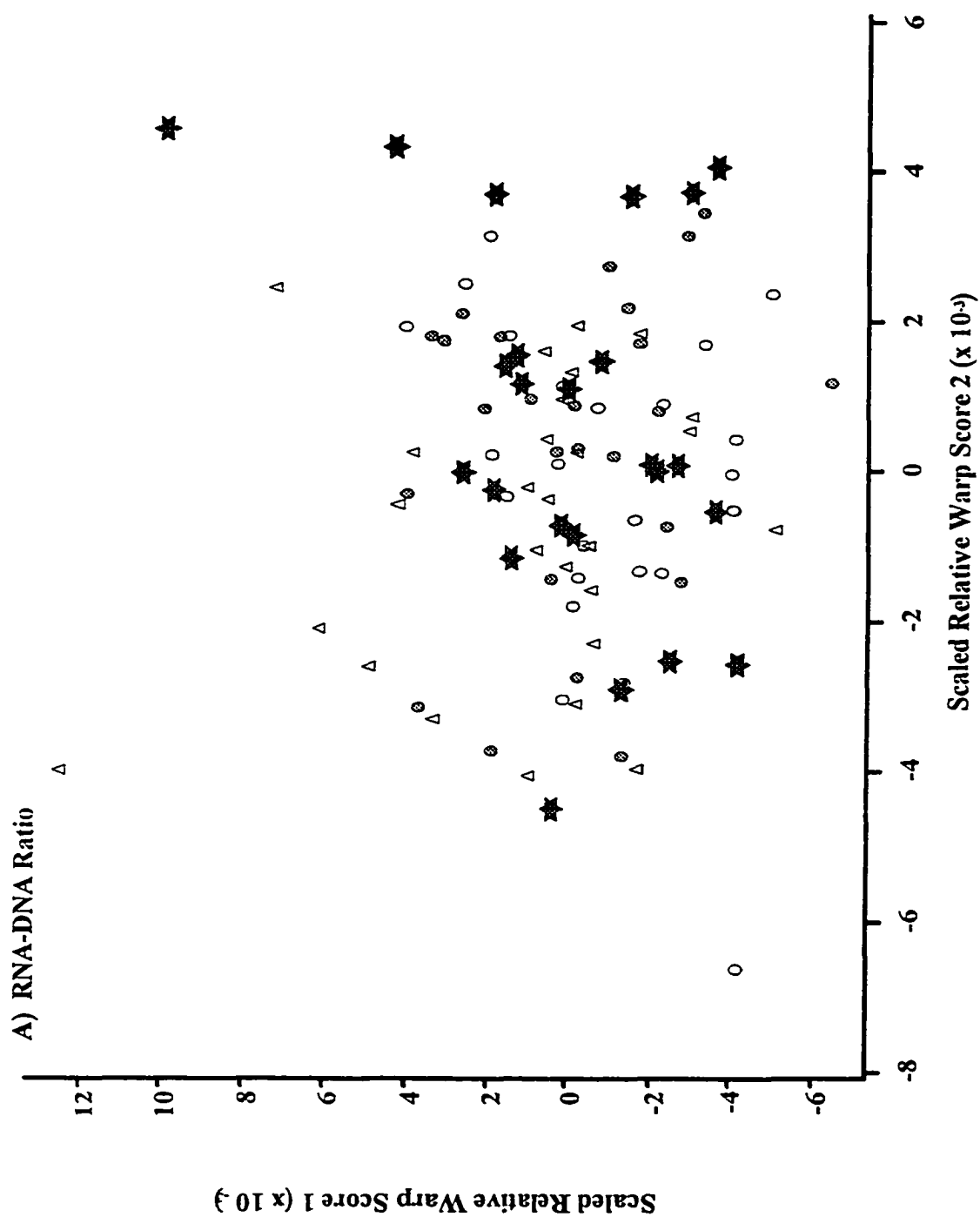
The first two relative warps (principal components) only explained 24% of the total sample variance, and the first five only accounted for 44% of the total variance. Plotting of the first three scaled relative warp scores revealed no ordination of the samples in relation to RNA concentration, ETS activity or length (Figure 4.5-4.7). If the shape of the larvae was influenced by their present condition, there should be an ordination of individuals with respect to their biochemical condition. In each plot, the categories of biochemical indicators of condition greatly overlap and the analysis of the relative warps indicates that there is no detectable difference in the overall shape of the larvae based on biochemical condition.

## **DISCUSSION**

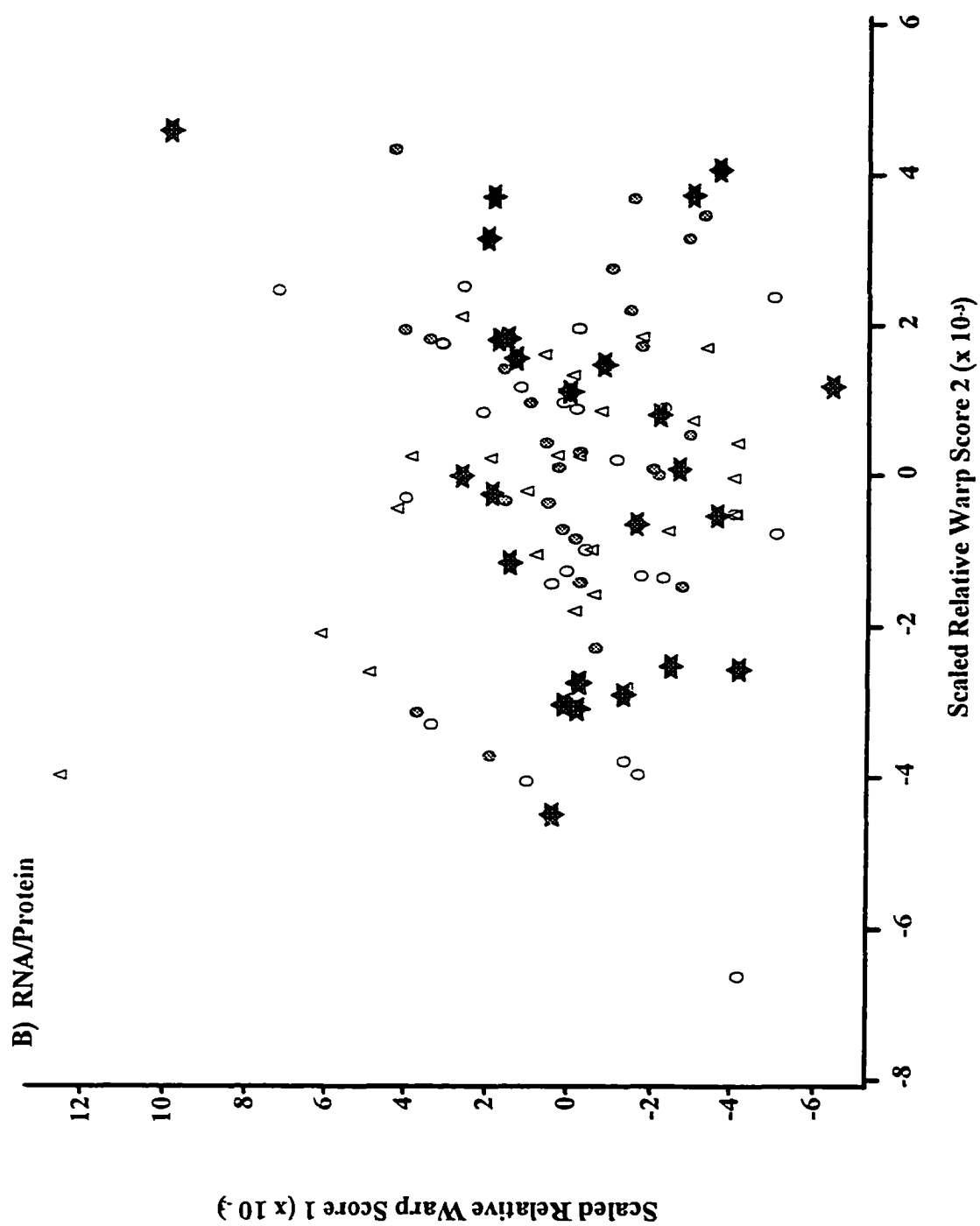
Thin-plate spline relative warp analysis has been used to successfully quantify and categorize the overall body shape of various organisms including larval fish of varied condition (Bookstein, 1991; Rohlf, 1992; Horn, 1996). In this study however, the technique had no predictive capabilities nor the ability to separate larvae of varied condition. The lack of detectable difference can be explained by several hypotheses: (1) There is no change in larval shape in response to differences in condition; (2) The

**Figure 4.5: Plot of scaled relative warp score 1 versus scaled relative warp score 2 based on  $\alpha=0$ . Different symbols represent grouping based on the quartiles of the observed biochemical measures of condition. A) RNA-DNA values, B) RNA Protein<sup>-1</sup> ( $\mu\text{g RNA/mg Protein}$ ), C) ETS/DNA ( $\mu\text{l O}_2/\text{min}/\mu\text{g DNA}$ ), D) ETS/Protein ( $\mu\text{l O}_2/\text{min}/\text{mg protein}$ ), E) Standard length (mm).  $\star=0$  to 25<sup>th</sup> percentile,  $\bullet=25^{\text{th}}$  to 50<sup>th</sup> percentile,  $\bigcirc=50^{\text{th}}$  to 75<sup>th</sup> percentile and  $\triangle=75^{\text{th}}$  to 100<sup>th</sup> percentile.**

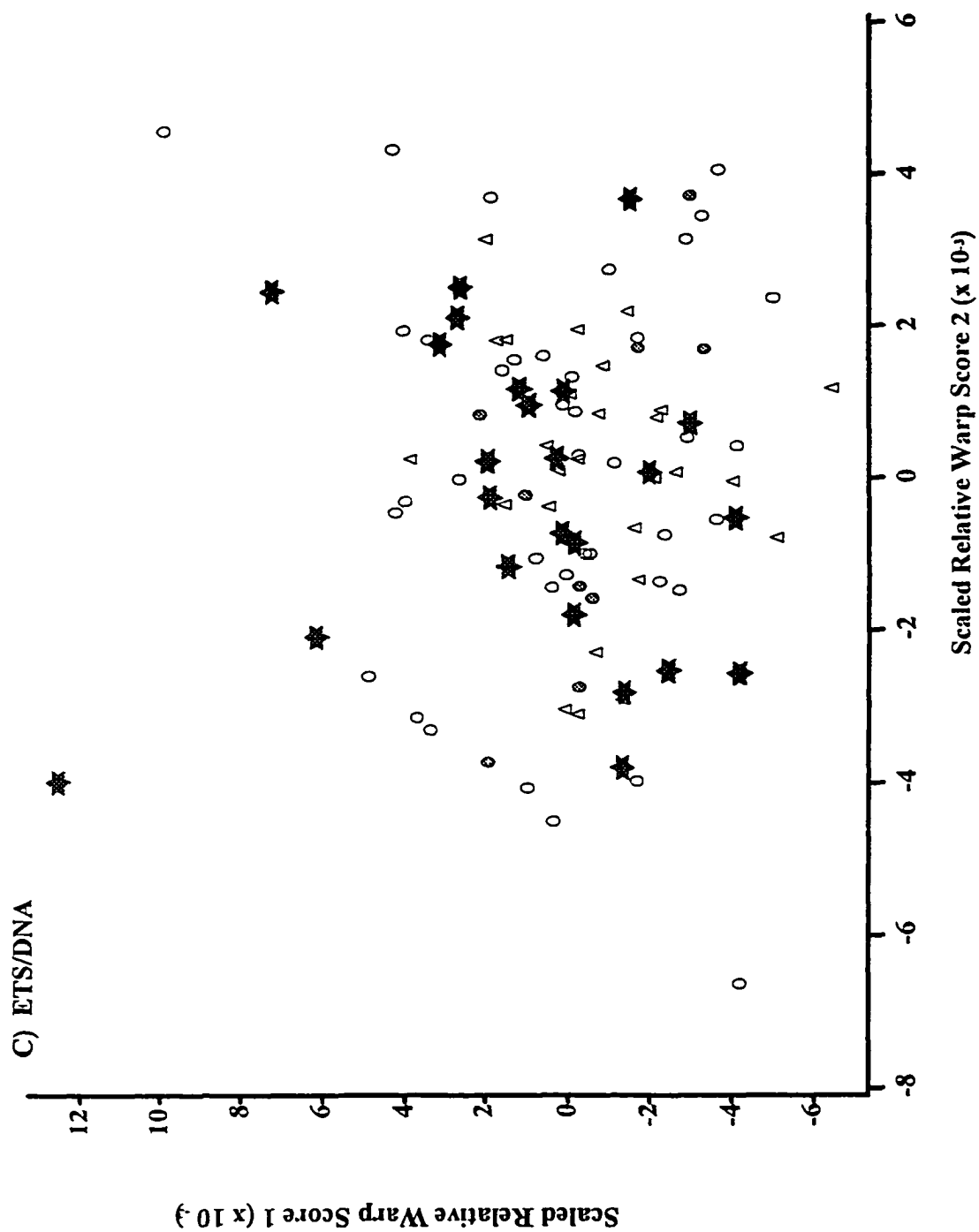




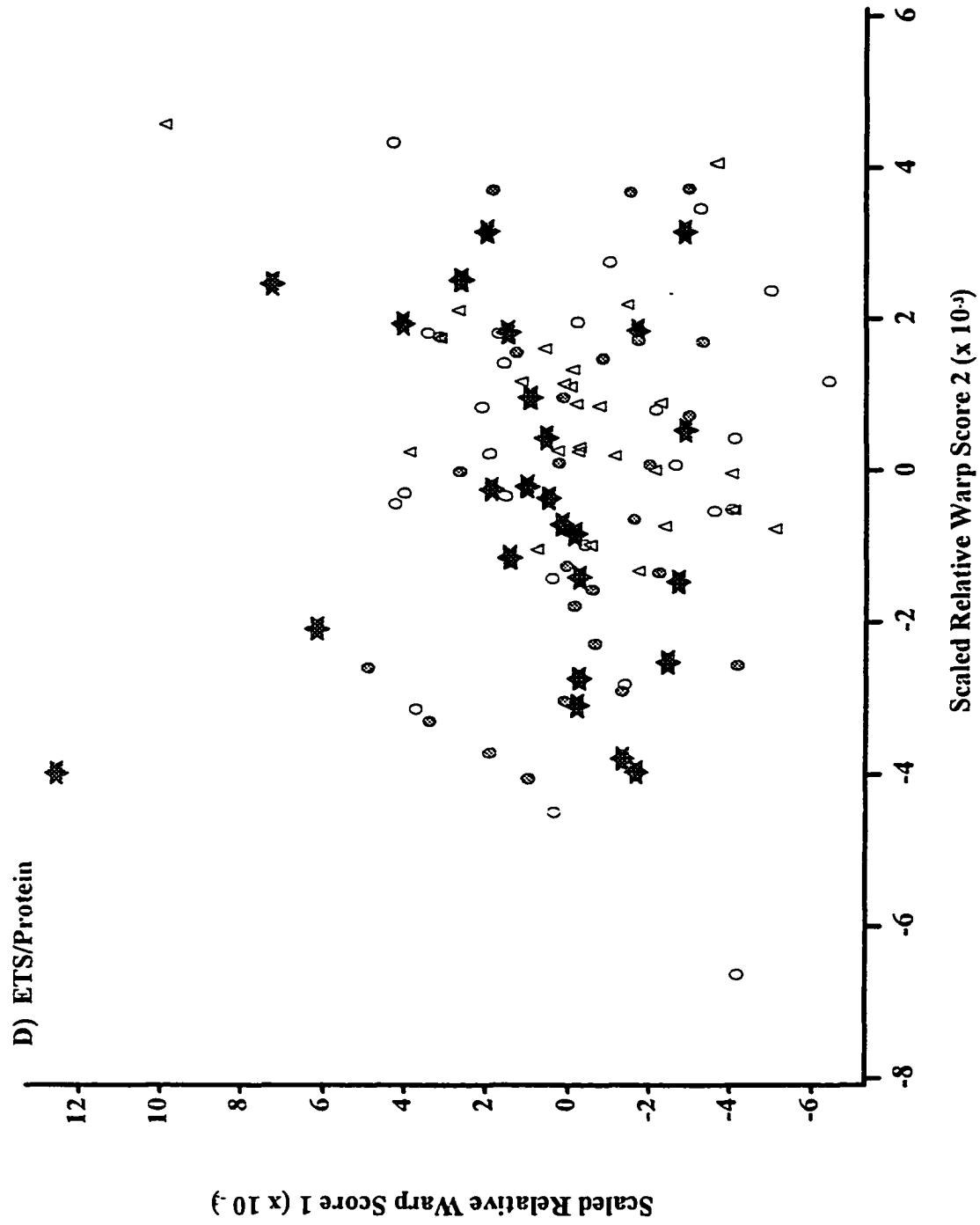
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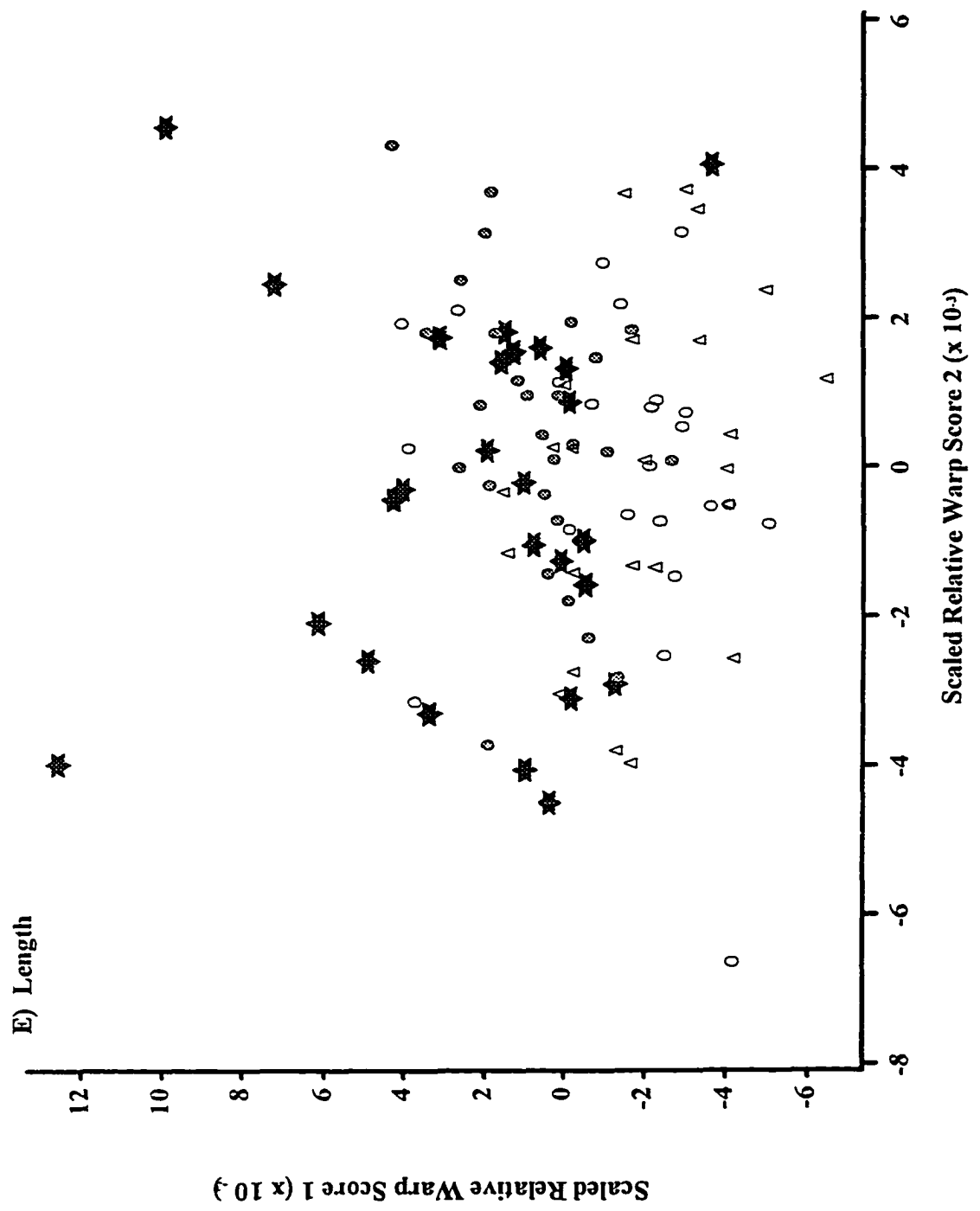
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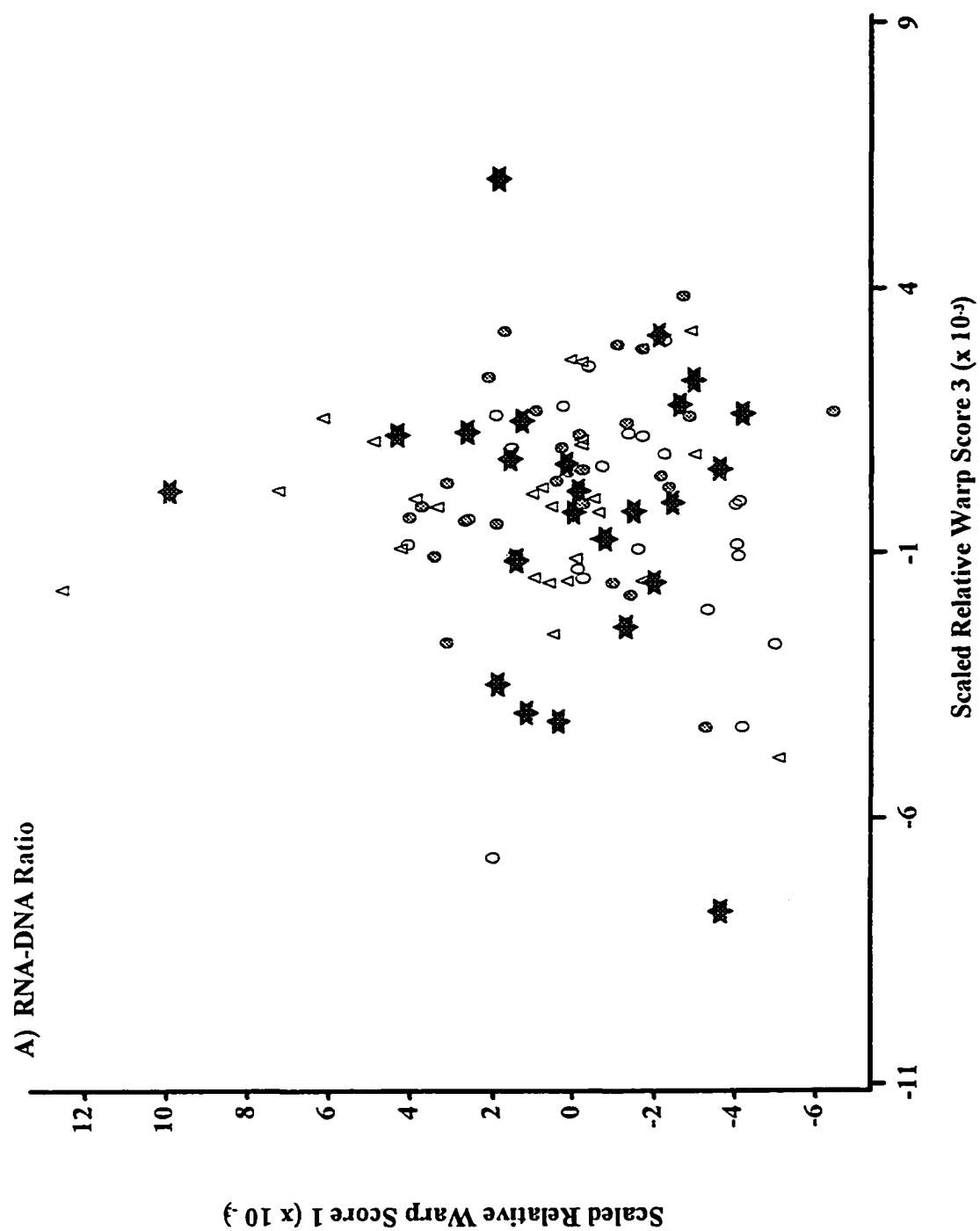
(Fig. con'd.)



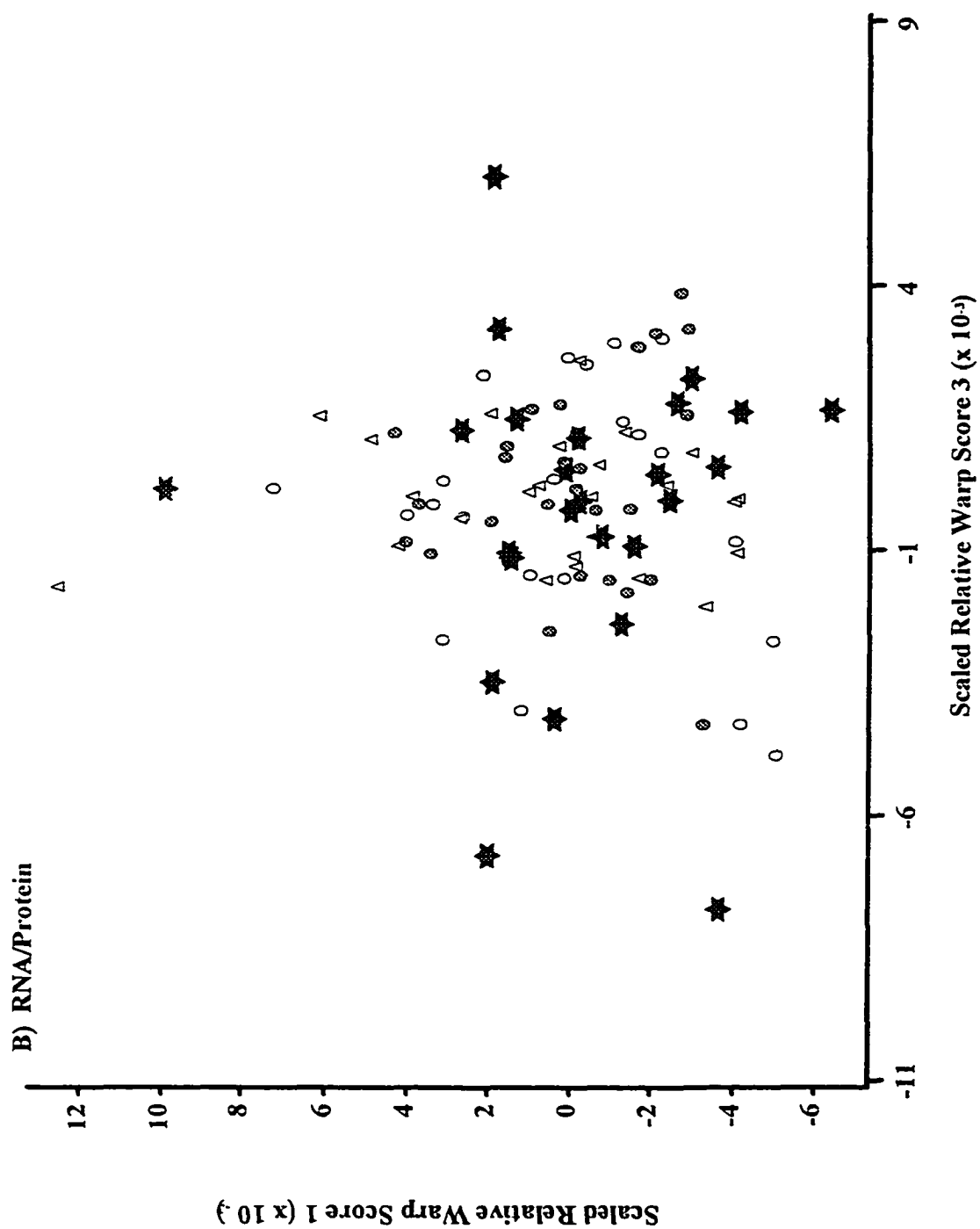
(Fig. con'd.)



**Figure 4.6: Plot of scaled relative warp score 1 versus scaled relative warp score 3 based on  $\alpha=0$ . Different symbols represent grouping based on the quartiles of the observed biochemical measures of condition. A) RNA-DNA values, B) RNA Protein<sup>-1</sup> ( $\mu\text{g RNA/mg Protein}$ ), C) ETS/DNA ( $\mu\text{l O}_2/\text{min}/\mu\text{g DNA}$ ), D) ETS/Protein ( $\mu\text{l O}_2/\text{min}/\text{mg protein}$ ), E) Standard length (mm).  $\star=0$  to 25<sup>th</sup> percentile,  $\bullet=25^{\text{th}}$  to 50<sup>th</sup> percentile,  $\bigcirc=50^{\text{th}}$  to 75<sup>th</sup> percentile and  $\triangle=75^{\text{th}}$  to 100<sup>th</sup> percentile.**

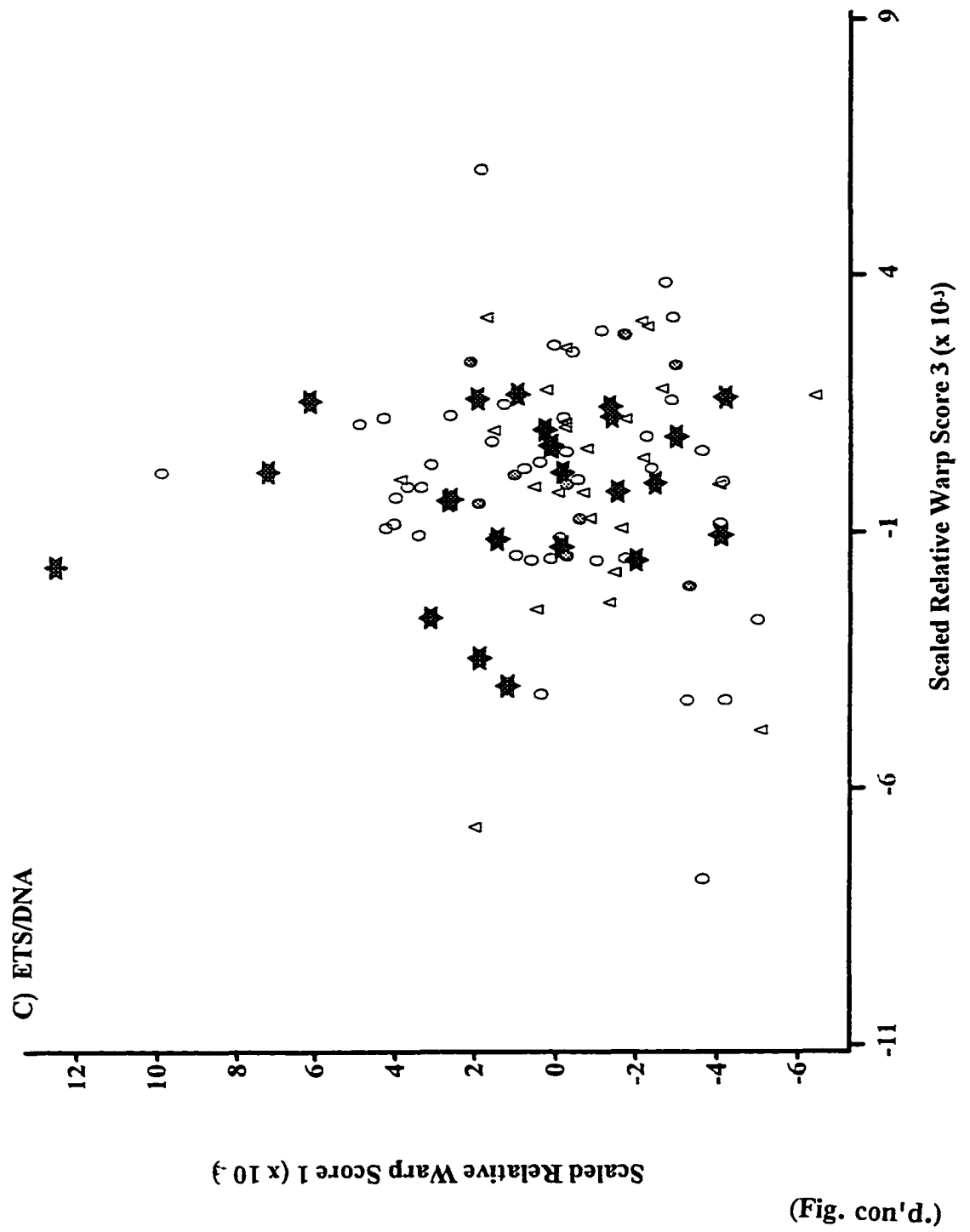


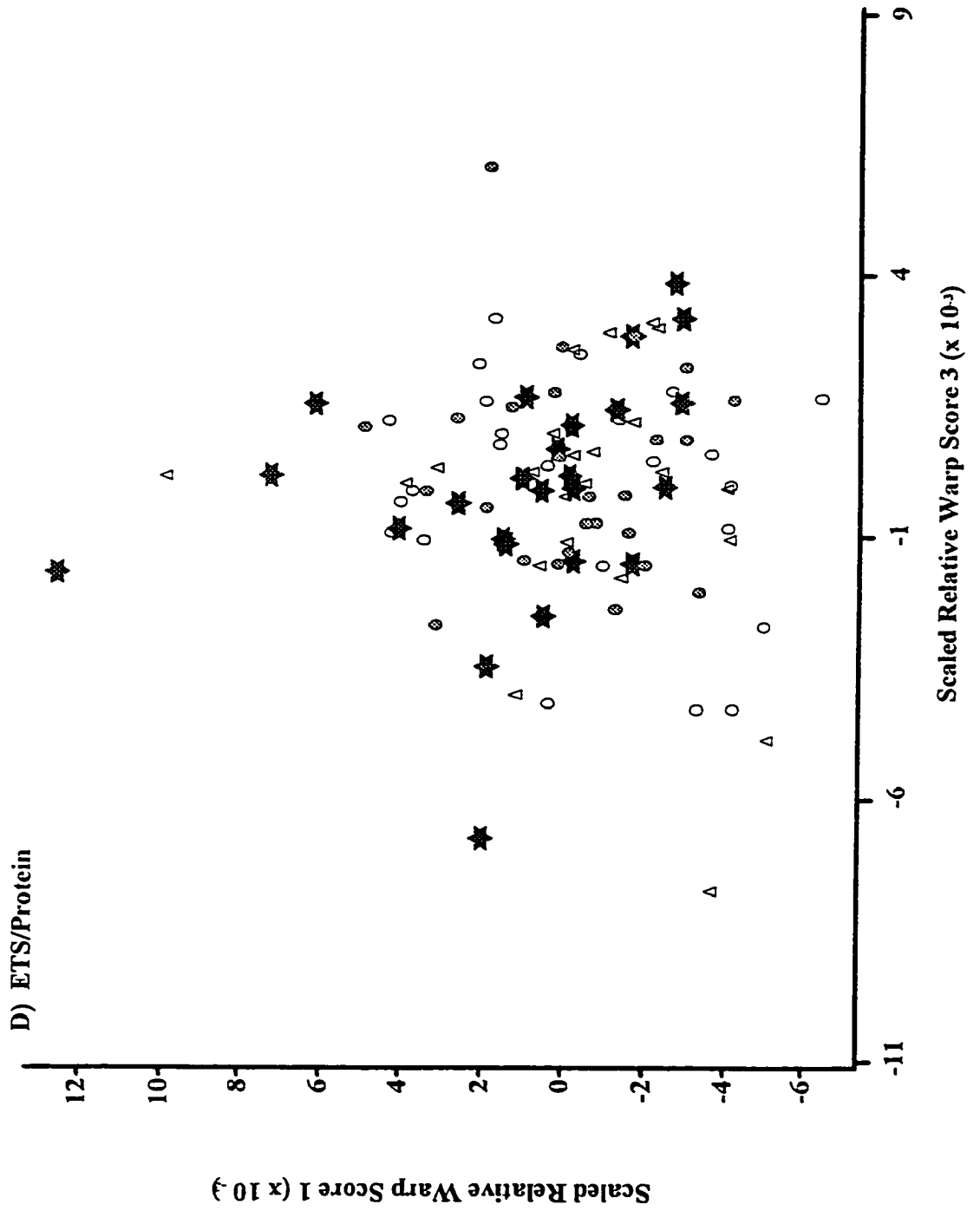
(Fig. con'd.)



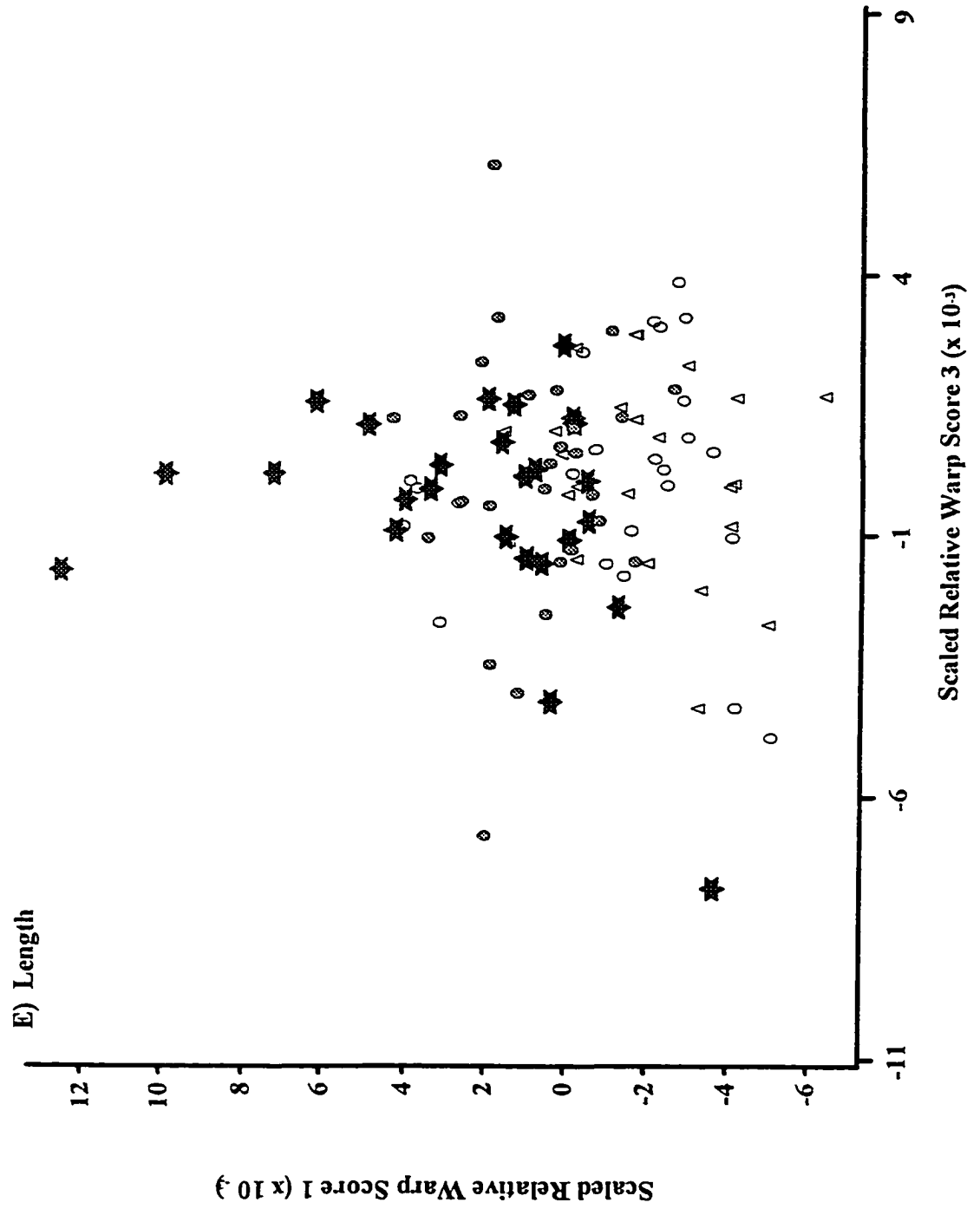
(Fig. con'd.)



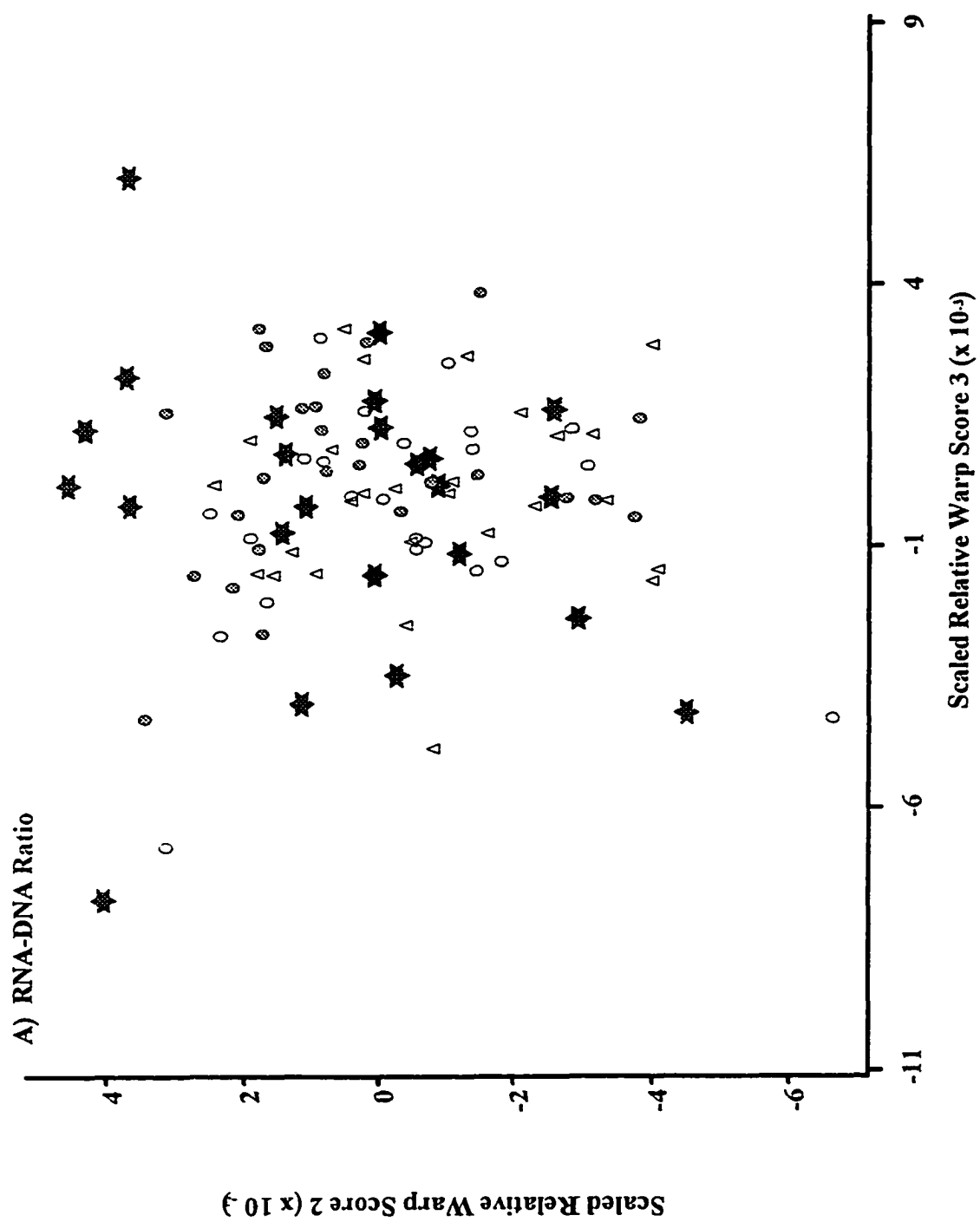




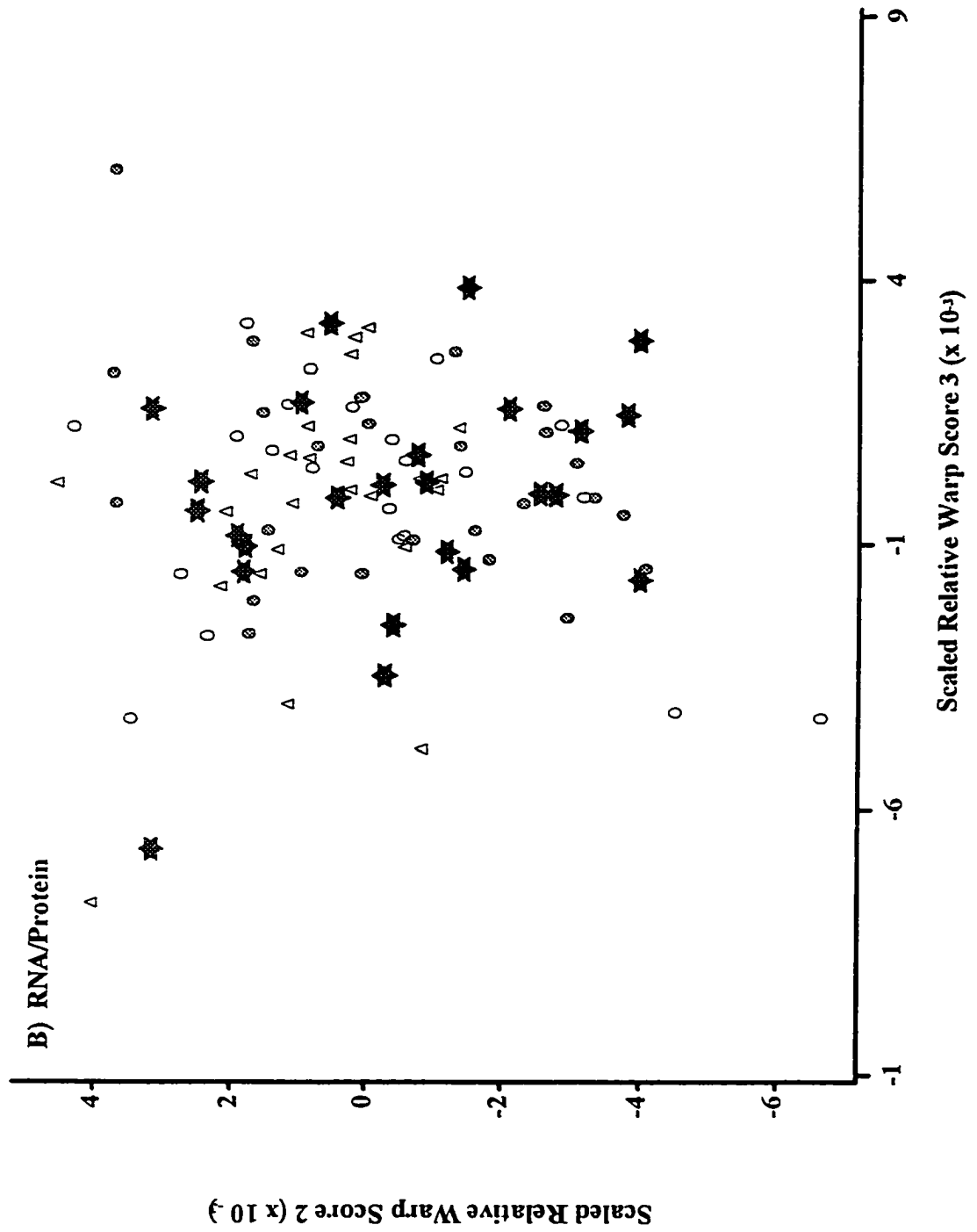
(Fig. con'd.)



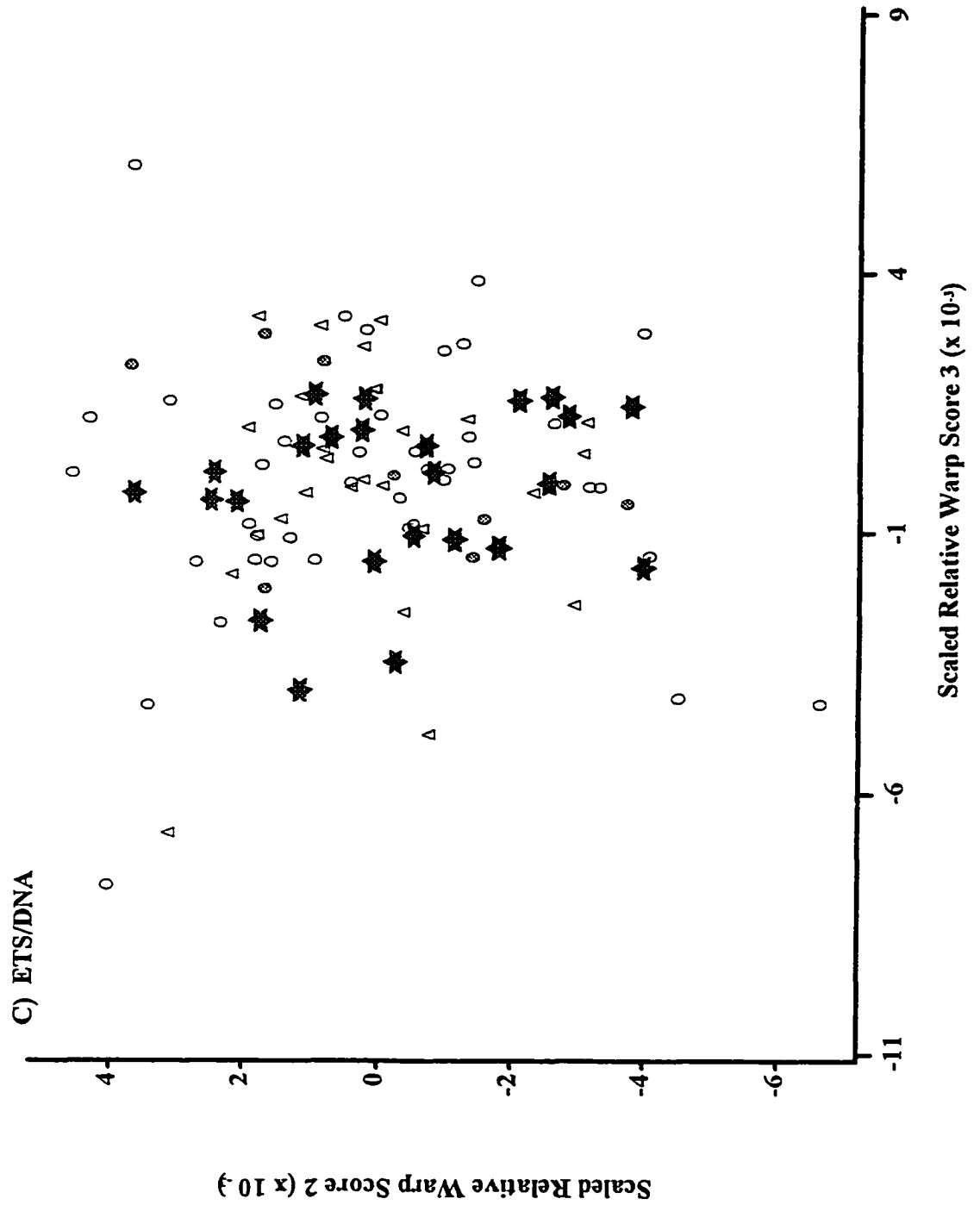
**Figure 4.7: Plot of scaled relative warp score 2 versus scaled relative warp score 3 based on  $\alpha=0$ . Different symbols represent grouping based on the quartiles of the observed biochemical measures of condition. A) RNA-DNA values, B) RNA Protein<sup>-1</sup> ( $\mu\text{g RNA/mg Protein}$ ), C) ETS/DNA ( $\mu\text{l O}_2/\text{min}/\mu\text{g DNA}$ ), D) ETS/Protein ( $\mu\text{l O}_2/\text{min}/\text{mg protein}$ ), E) Standard length (mm).  $\star=0$  to 25<sup>th</sup> percentile,  $\bullet=25^{\text{th}}$  to 50<sup>th</sup> percentile,  $\bigcirc=50^{\text{th}}$  to 75<sup>th</sup> percentile and  $\Delta=75^{\text{th}}$  to 100<sup>th</sup> percentile.**



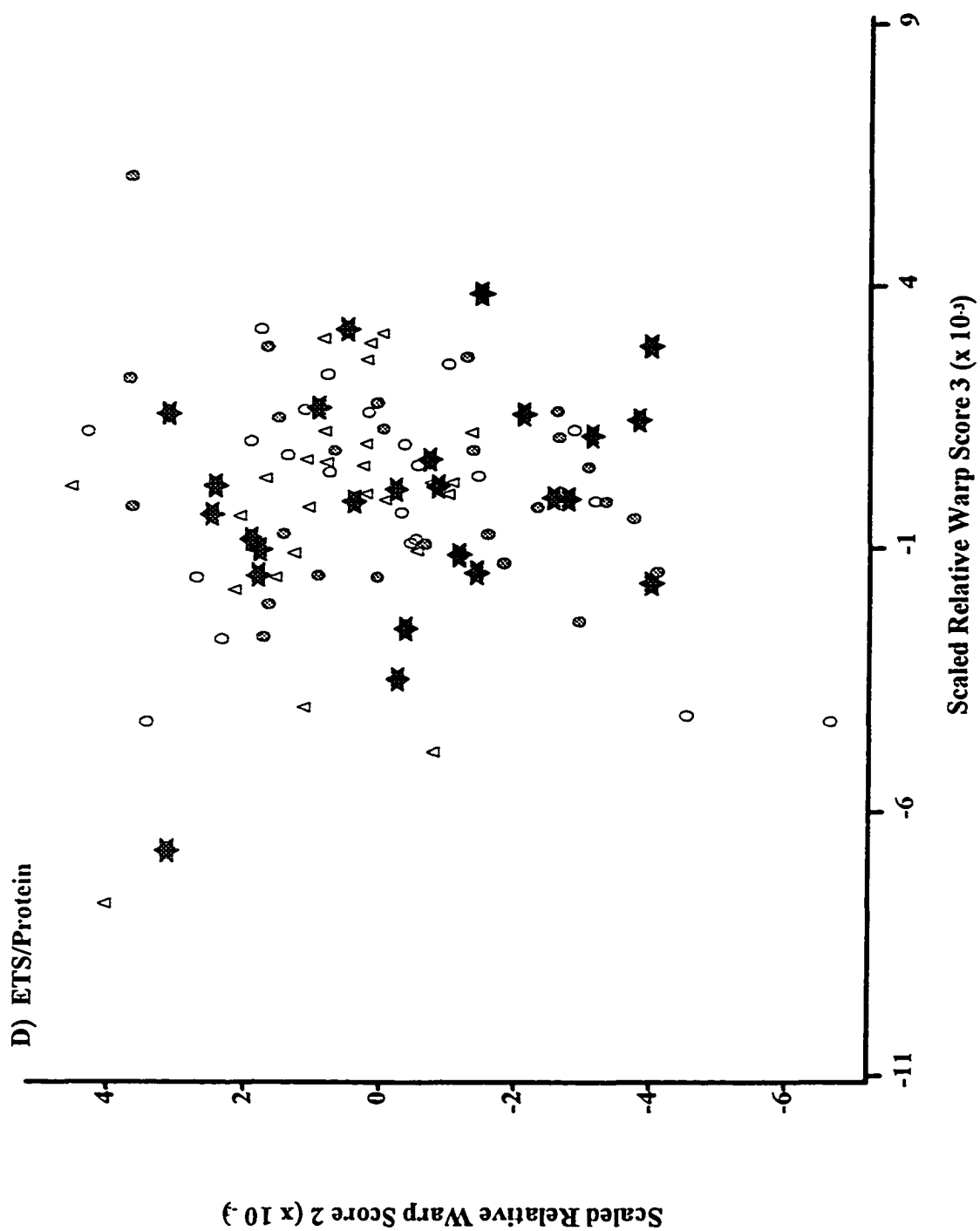
(Fig. con'd.)



(Fig. con'd.)

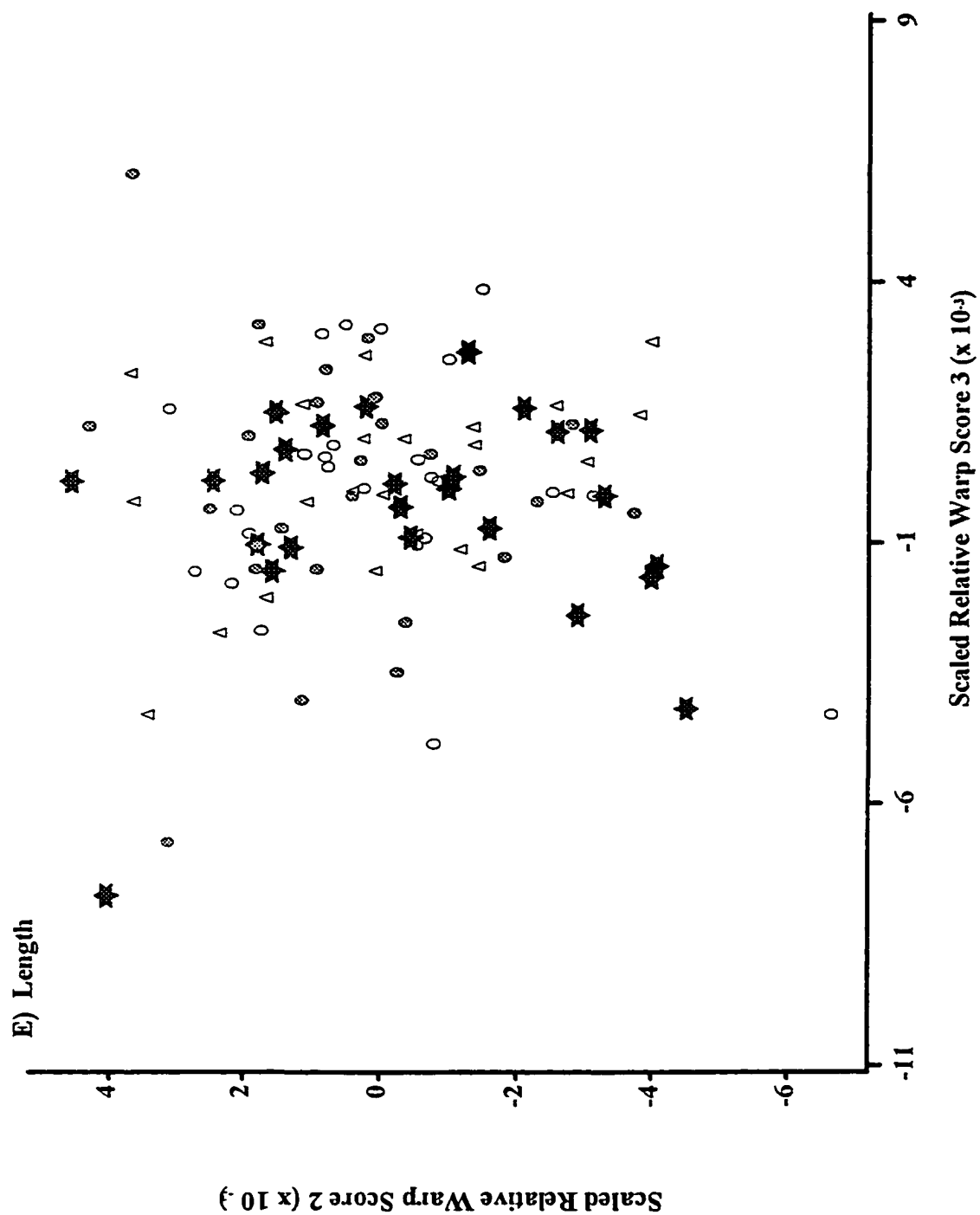


(Fig. con'd.)



(Fig. con'd.)





stress was of insufficient magnitude or duration to result in significant shape changes.

(3) The power of thin-plate spline relative warp techniques and other morphological measures of condition lie in the ability to detect similar but distinctly shaped groups rather than the separation along a continuum of shapes.

There is ample research that indicates that differences in the shape of a larva can be used as an indicator of the relative condition. Theilacker (1978) showed that a series of 5 different ratios of body measures could correctly discriminate between fed and unfed jack mackerel (*Trachurus symmetricus*) over 85% of the time. Yin and Blaxter (1987) found that gut height/myotome height was useful in the separation of starved and unstarved cod (*Gadus morhua*) and flounder (*Platichthys flesus*). McGurk (1985) used principal component analysis on 6 morphometric measurements (head width, body depth at the anus and pectoral fins, eye diameter and standard and total length) to correctly identify laboratory starved herring (*Clupea harengus*) larvae. Setzler-Hamilton and Cowen (1993) used relative condition,  $K_n$ , (a form of Fulton's  $K$ ,  $K_n = \text{weight}/\text{length}^b$ ) to assess the condition of field collected larval striped bass (*Morone saxatilis*) and found that the frequency of poorer condition larvae reasonably reflected the yearly estimate of juvenile abundance. Based on the volume of work on several different species, it would seem unlikely that the morphology of larval menhaden would be unaffected at least to some extent by the nutritional condition.

Ferron and Leggett (1994) report the latency for morphological detection of environmental stress as 2-3 days based on laboratory studies. Theilacker (1978) detected significant differences in morphology after 2 days of starvation. Wright and

Martin (1985) found that the latency of a morphological response to stress increased with age. Horn (1996), using thin-plate spline relative warp analysis, found only minimal differences in the shape of razorback sucker larvae after 14 days of poor feeding conditions when compared with well fed larvae. These morphological differences were more pronounced after 22 days.

The hypothesized source of the condition variation in this study, atmospheric frontal passage, had a duration of approximately 1-2 days. While this duration may be sufficient to generate morphological differences in young larvae, it does not appear to have been sufficient to induce changes in the older larvae that were examined in this study. Additionally, the effect of atmospheric frontal passage simply enhanced existing and presumably adequate feeding conditions, thus the comparisons in this study did not include the extreme contrast in available food (well fed vs. starved) of many of the laboratory based studies (Theilacker, 1978; Wright and Martin, 1985; Yin and Blaxter, 1987; Horn, 1996). It is also reasonable to expect that the biochemical difference in condition examined in the previous chapter would be detected prior to any morphological difference. The biochemical measures of condition examined in this study have a latency of 1 day (Ferron and Leggett, 1994). In a biological sense, the progression of condition indices in response to an environmental stress should be biochemical changes, followed by histological changes, and finally morphological changes. However, this does not imply a lagged morphological response. If the stress is of short duration it seems probable that a morphological change would not occur. It is quite likely that the passage of atmospheric fronts, while significantly affecting the

condition of the larvae reflected by biochemical measures, were not of sufficient duration or intensity to also affect larval morphometrics.

Thin-plate spline relative warp analysis and other methods of shape analysis have been used to distinguish between morphologically similar but distinct species or groups of a single species. The examples in Bookstein (1991) include the separation of two species of ostracods (Abe et al., 1988), describing the growth of the cranium by analyzing 8 distinct ages of rats and comparison of the facial features of children with and without Aperts syndrome. Rohlf (1992) used an example of 8 species of mosquito from the genus *Anopheles* in which morphometric analysis on the venation patterns of the wings was used to separate the species. Horns' (1996) study of the condition of razorback sucker larvae examined groups of larvae reared at specific ration levels, therefore creating distinct groups of larvae. Many of the field and laboratory studies using ratio based condition indices also do comparisons of distinct groups. The studies examining the applicability of morphometric condition indices are experimentally bound to comparisons among groups fed different rations and commonly of identical age (Theilacker, 1978; Wright and Martin, 1985; Yin and Blaxter, 1986; Horn, 1996). Additionally, many field-based studies in which morphometric variation was determined to be significant are based on collections made at environmentally distinct areas. For example, Theilacker (1986) found the condition of jack mackerel larvae collected in the open ocean was lower than larvae collected along islands and banks off the coast of California. McGurk et al. (1993) attributed the variation in morphological condition of Pacific herring (*Clupea pallasii*) larvae to specific cohorts that had embryonic exposure

to temperature extremes and dessication rather than feeding conditions. Burke et al. (1993) examined Atlantic croaker (*Micropogonias undulatus*) larvae in relation to a pollution gradient and concluded that the observed differences in morphology were the result of long-term residence in the polluted waters. Frank and McRuer (1989) determined that haddock (*Melanogrammus aeglefinus*) larvae in deep, stratified waters had a higher condition (Fulton's K) than larvae collected in shallow well mixed area.

In the present study, the larvae were of varied size and presumably ages, thus the morphology would respond to nutritional changes differentially based upon age and/or size (Wright and Martin, 1985). This may have created a continuum of responses that may appear as variation rather than a defined trend in shape change. Furthermore, the larvae collected for this research do not necessarily represent long term environmentally distinct groups such as near-shore-offshore or polluted-unpolluted which create morphologically distinct groups (Theilacker, 1986; Burke et al., 1993). Consequently a combination of the relatively long latency period of morphological condition indices, short duration of the environmental alteration, and the varied size and age of the larvae probably precluded a morphological detection of nutritional variation of the menhaden larvae in this study.

Because there was a significant biochemical response of condition indices to the atmospheric frontal passage, but no significant morphological response, the utility of morphological condition indices to detect changes in condition should be critically explored before further use. Houde (1986) stressed the importance of subtle alterations in growth and mortality rates in the ultimate determination of year-class strength. This

study indicates that morphometric indices are unable to detect these subtleties.

Additionally, there is also only limited evidence of wide spread starvation in the field, and in this study there was no evidence of wide-spread starvation of menhaden larvae. It is unlikely that a condition index that may require 14 to 22 days of severely depressed feeding (Horn, 1996) would be of ecological utility under anything but the most extreme of circumstances. It is also interesting to note that a majority of the studies documenting starvation in the field are based on morphometric condition indices (e.g. Theilacker, 1986; Govoni et al., 1983). If the variation in the biochemical condition of menhaden larvae observed in this study are responsible for subsequent changes in growth or survival of the larvae, then it is likely that the biochemical measures of condition, rather than the morphological indices, are a much more appropriate unit of measure for determining the potential contribution of a larval cohort to year class strength.

## **CHAPTER 5: SUMMARY AND CONCLUSIONS**

### **SUMMARY**

The purpose of this study was to examine the affect of episodic wind events on the condition of menhaden larvae. The development of a highly sensitive nucleic acid assay and associated techniques allowed the utilization of four measures of condition (three biochemical, one morphometric) on individual menhaden larvae. These assays were then used in an examination of the effect of atmospheric frontal passage on the relative health of the larvae.

Central to the completion of this study was the development and integration of the biochemical assays used to measure condition. The nucleic acid assay described in Chapter 2 used the non-specific nucleic acid fluorochrome thiazole orange in combination with RNase to accurately detect and quantify nucleic acids at concentrations as low as 3.3 ng/ml for DNA and 15.7 ng/ml for RNA. This assay procedure resulted in near 100% recovery of nucleic acid standard spikes added to larval homogenate. RNase concentrations of 6.67  $\mu$ g/ml and an incubation of 30 min at 29°C were found to be sufficient to completely digest the RNA present in a sample while not affecting the DNA present in the sample. Trials using DNase to remove the DNA present in sample homogenate proved unsuccessful. The addition of divalent cations as co-factors increased the digestive capabilities of DNase but decreased the sensitivity of the thiazole orange to unacceptable levels. It was determined that sonication was the better method for extracting the nucleic acids from larval tissue when compared to grinding and that the crude tissue homogenate could be fast frozen

in liquid nitrogen for 24 h without loss of nucleic materials. In fact, the fluorescence of the DNA was increased after freezing, while the fluorescence of RNA was unaffected. The addition of proteinase-K at concentrations of 0.375 mg/ml and incubating at 29°C for 30 min increased the total fluorescence of the larval homogenate by 100%. The increase in sample fluorescence after freezing the homogenate was most likely a result of the disruption of coagulations of cellular materials by ice crystals allowing the fluorescent dye greater accessibility to the nucleic acids. The increase in homogenate fluorescence after the addition and incubation of proteinase-K was the result of the removal of nucleoproteins, making the nucleic acid more accessible to the dye.

The nucleic acid assay developed in this study is sensitive enough to detect small amounts of both DNA and RNA; it is compatible with assays for electron transport enzyme activity and protein concentration. Additionally the sonication procedure allows for the recovery of the otoliths.

Chapter 3 was an examination of the condition of field collected menhaden larvae in relation to atmospheric frontal passage and the progression of the spawning season. There are conflicting reports on the impact of the increased winds associated with meteorological events on the feeding and growth of fish larvae, Maillet and Checkley (1991) report decreased growth in association with increased winds while MacKenzie et al. (1994) predict an increase in larval ingestion rates.

To access the influence of episodic wind events on the condition of menhaden larvae, late-stage larval were collected during the winter months of 1993-1994 and



1994-1995. Collections were made daily at Port Fourchon, Louisiana using a light trap. The condition of the larvae was assessed using biochemical measures of condition including RNA DNA ratios, ETS activity and protein concentration.

A total of 1,349 menhaden larvae were collected and biochemically analyzed. Field collected menhaden larvae were in good condition and there was no indication of wide spread starvation; less than 3% of the larvae had RNA:DNA values below the hypothesized critical level of 2. There was good correlation among the series of biochemical measures examined. ETS measures of condition correlated well with the more extensively researched RNA based condition indices (RNA:DNA and RNA:protein). RNA:DNA values and DNA or protein corrected ETS activity were not strongly correlated to length because the samples were late stage larvae which generally do not exhibit a length dependency of condition.

There was only weak correlations between larval condition and wind speed and no evidence of the dome-shaped relationship predicted by MacKenzie et al. (1994). There was a slight positive correlation between larval condition and the southerly wind component, while the correlation between condition and the westerly wind component was negative. There was no relationship between the condition indices measured and water or air temperature.

Larvae collected during periods classified as pre-frontal had higher condition indices when compared to larvae collected during post- and inter-frontal periods. For RNA based measures of condition, the pre-frontal levels of condition were also greater than cold-front levels of condition. The variation in condition observed

between year of collection and among months within a collection year can be explained by the frequency of pre-frontal periods. The increase in larval condition associated with the southerly wind dominated pre-frontal periods is likely to be a result of the increased encounter rate between larvae and their prey due to turbulent mixing.

In an attempt to develop a rapid low-cost alternative to the biochemical measures of condition, the applicability of novel morphometric analysis was examined in Chapter 4. This technique used thin-plate spline relative warp analysis to quantify the overall shape of the larvae which was then related to the biochemical measures of condition described in Chapter 3.

Thin-plate spline relative warp analysis was unable to detect or characterize morphological changes associated with the variation in biochemical condition of the larvae. It is likely that atmospheric frontal passage is of too short duration and too low intensity to generate a morphological change in response to alterations in the feeding conditions experienced by the larvae.

## CONCLUSIONS

The condition of menhaden larvae collected in the estuary is influenced by the frequency of atmospheric frontal passage. Larval condition increased in association with the southerly wind dominated pre-frontal periods. This increase is likely due to the increase in encounter rates between the larvae and their prey. While most larvae were in good condition, regardless of weather classification, the subtle differences in condition may lead to increased larval stage duration for lower condition larvae. The

increase in stage duration will subject the larvae to the higher larval mortality rates for a greater period of time. The morphometric measure of condition examined in this study was unable to detect these subtle changes. Guillory et al. (1995) summarized a series of menhaden recruitment predictive models and concluded that the greatest year-class should be associated with cold, dry winters as opposed to warm, wet winters. Cold, dry winters would result from a high frequency of rapid cold front progression.

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## **VITA**

Michael John Burger was born in Spokane, Washington, on September 24, 1962. From the 2<sup>nd</sup> grade on, he was raised in Beaverton, Oregon. In the spring of 1981, he graduated from Jesuit High School located in Portland, Oregon. He received a bachelor of science degree in Biology from Oregon State University in May, 1986. He worked as a laboratory technician at the Hatfield Marine Science Center from March, 1986 to June, 1989. He began his graduate career at the University of Delaware in September, 1989. He received his master of science in 1992 from the College of Marine Studies, University of Delaware. Michael began a doctoral program at Louisiana State University in September, 1992 and will receive his doctor of philosophy in Oceanography and Coastal Sciences in May, 1998.

DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Michael John Burger

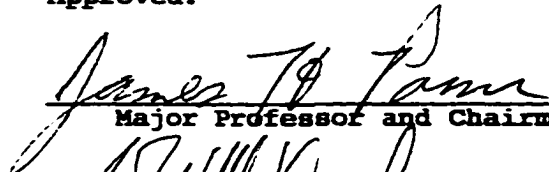
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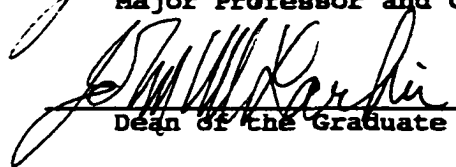
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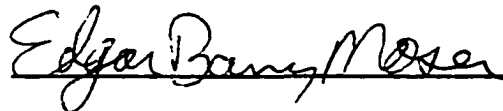
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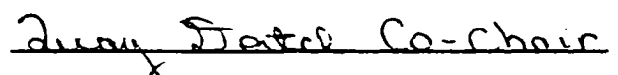
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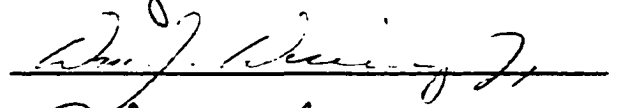
  
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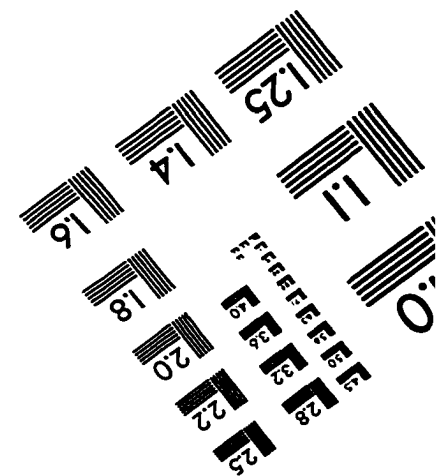
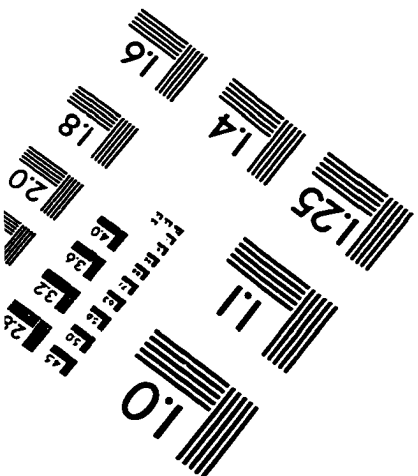
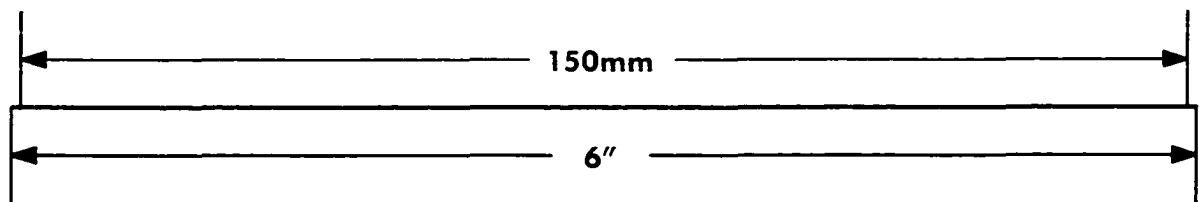
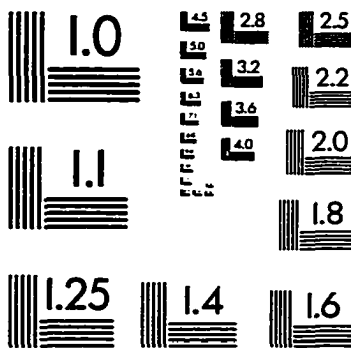
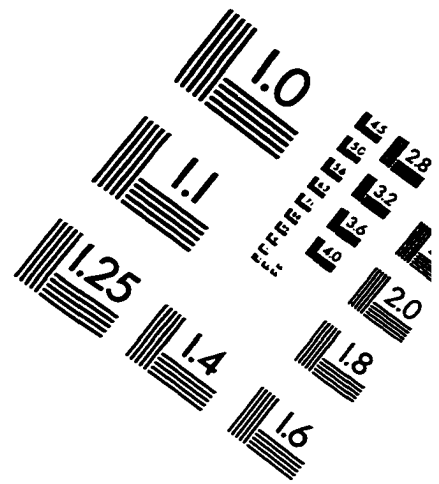
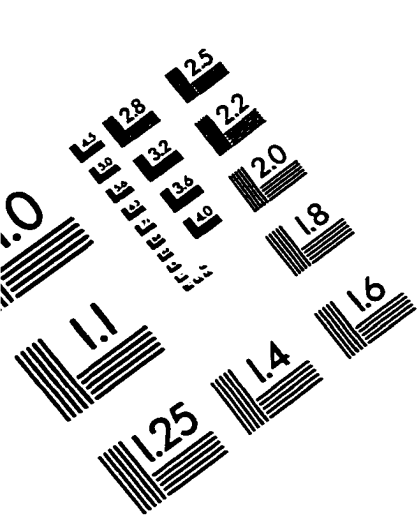
  
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